MOLECULAR MODELLING EXERCISES

EXERCISE 20.3 RITONAVIR

INTRODUCTION

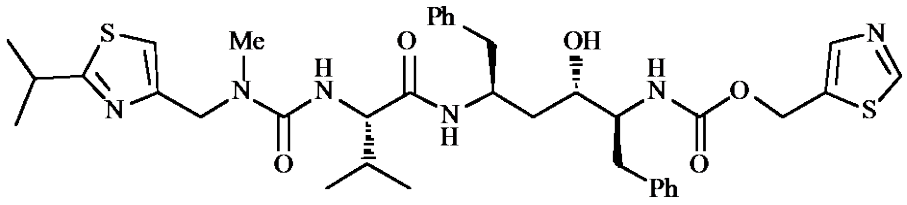

Figure 1 Ritonavir.

Ritonavir (Fig. 1) was one of the first HIV protease inhibitors (PI) to reach the market. The lead compound for the protease inhibitors was a peptide structure, and the successful design of the protease inhibitors involved various peptidomimetic strategies aimed at diminishing peptide character and increasing oral bioavailability. Despite this, ritonavir is a relatively large and flexible molecule with a low oral bioavailability. Because of the number of rotatable bonds, there are a large number of possible conformations and it is impossible to predict what the active conformation might be. The only way to achieve this is to crystallise the target protein with ritonavir bound to the active site and use that to identify the active conformation.

In this exercise, you will study the flexibility of ritonavir by identifying the number of rotatable bonds. You will then identify the active conformation from a crystal structure of the protein with bound ritonavir. Finally, you will identify specific binding interactions between ritonavir and amino acid residues in the binding site. Ritonavir interacts with the catalytic aspartate residues. It also has substituents that bind to five binding subsites, while the main chain can form additional binding interactions.

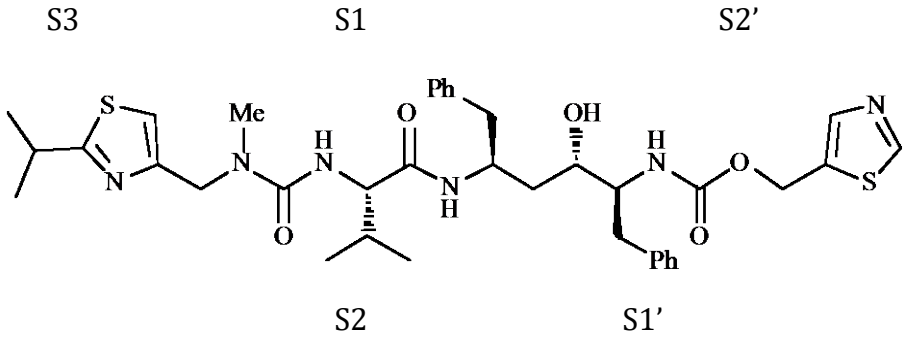

Figure 2 Binding pockets or subsites (S3, S2, S1, S1' and S2')) that are occupied by groups present in ritonavir.

INSTRUCTIONS

It is suggested that you try out the following instructions yourself before following the more detailed **Procedures** that follow. You may find the file entitled **Common Operations for ChemBio3D/Chem3D** a useful guide on how to carry out various operations. A ChemDraw file for ritonavir is available in the ChemDraw folder.

PART A

Create the energy-minimised 3D structure of ritonavir and identify the number of rotatable bonds that are present in the structure.

PART B

*Download the crystal structure of the protease enzyme with bound ritonavir (pdb 1HXW).
*Examine the shape of the enzyme and the location of the inhibitor.

PART C

*Identify various components of the complex.

PART D

*Locate the binding site and identify amino acids in that vicinity.
*Highlight the position of the catalytic amino acid residues Asp-25 and Asp-25'

PART E

*Extract the structure of ritonavir and study its conformation.
*Compare the active conformation of ritonavir with the energy-minimised conformation of ritonavir obtained in Part A.

PART F

*Create a model binding site that identifies the amino acids present.
*Identify hydrogen bond interactions with Asp-29 and Asp-30'.
*Identify possible pi-pi interactions with Arg-8.

PART G

*Create a model binding site that allows you to add hydrogen atoms to ritonavir.
*Identify hydrogen bond interactions with the following amino acid residues; Asp-25 & Asp-25'; Asp-29; Gly-48; Gly-27; Asp-30'; and Ile-50 & Ile-50'.

PART H

*Compare your findings with the binding predictions made by the PoseView image on the pdb web site.

PROCEDURES

There are various approaches that you can use to tackle these molecular modelling exercises. The following procedures illustrate how you might tackle this particular exercise, but they are not meant to be prescriptive. Note also that the results obtained may vary depending on the computer and the version of ChemBio3D/Chem3D used. For example, the specific conformations obtained from energy minimisation may differ, as may quantitative results such as steric energies. A ChemDraw file for ritonavir is available in the ChemDraw folder.

PART A) Structure of ritonavir

1. Create the energy-minimised 3D structure of ritonavir.

Open* **ChemBio3D or **Chem3D**.

*From the **File** menu *choose* **Open**, then find the ChemDraw file for ritonavir. *Select* it, then *click* on **Open**.

*Energy minimise the structure 

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The conformation shown in figure 3 is one of several possible conformations that might be formed. The steric energy of this conformation is given in the bottom window as 3.1 kcal/mol.

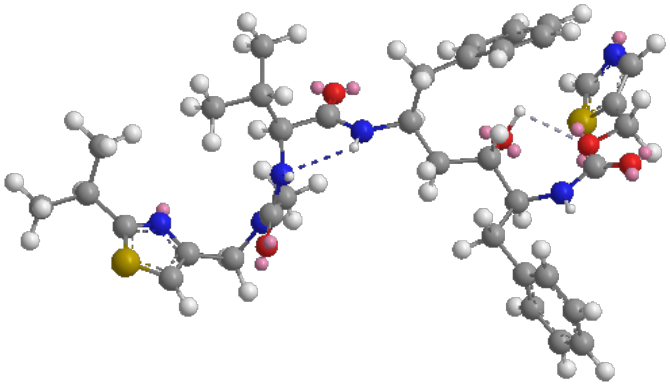


Figure 3 Energy-minimised conformation of ritonavir.

2. Determine the number of rotatable bonds in the structure.

*From the **Calculations** menu *choose* **Compute Properties**.

Expand* **Molecular Topology and *select* **Num Rotatable Bonds.** *Click* **OK**.

The number of rotatable bonds identified is given in the bottom window as 22.

Note that rotatable bonds in this context are defined as those that result in distinct differences in conformation. Rotatable bonds that only alter the relative positions of hydrogen atoms (e.g. C-OH, C-NH_2, C-CH_3 etc) are not included in the total. The rotatable bonds in ritonavir are coloured red in figure 4. The software program has also identified amide and urea bonds (coloured blue) as rotatable. However, these are not freely rotatable bonds and several other software programs would not identify them as such. Therefore, there are really 18 truly rotatable bonds in the structure. This is still a large number and exceeds the number identified by Veber in his rules relating to oral bioavailability (section 11.3 in the textbook). It also means that there is a vast number of possible conformations for ritonavir. Therefore, it is not easy to identify the most stable conformation. Even if we could, we cannot assume that the most stable conformation is the active conformation. The only sure way of identifying the active conformation is to study how the structure binds to the target binding site. This can be done by determining the crystal structure of the enzyme/ligand complex using X-Ray crystallography.

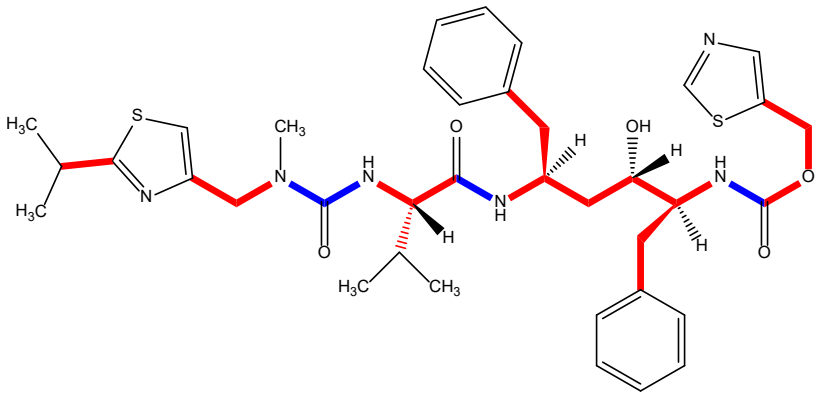


Figure 4 Rotatable bonds shown in red for ritonavir. Bonds shown in blue are not truly rotatable but have been identified as such by ChemBio3D/Chem3D.

PART B Analysis of the crystal structure for the HIV-1 protease/ritonavir complex.

1. Retrieve the crystal structure of the HIV-protease enzyme with bound ritonavir (pdb 1HXW).

The crystal structure can be downloaded from the protein data bank, and so you will need to be connected to the internet in order to do this.

*From the **Online** menu, *choose* **Find Structure from PDB ID**.

Enter* the PDB code for ritonavir bound to the protease enzyme (1HXW**).

Click* on **Get File and the protein will appear as a ribbon diagram, with the ligand present as a ball and stick model (Fig. 5).

2. Analyse the overall shape of the protein-ligand complex.

The protein is represented as a ribbon structure with a couple of light purple sections corresponding to alpha-helices, and dark blue sections corresponding to beta sheets. The pink regions are connecting regions that do not have a secondary structure. The ritonavir ligand is shown as a ball and stick model in the binding site.

Note the symmetry of the protease enzyme, which is made up of two protein subunits. The protein is relatively small which means that the ligand is clearly visible in the active site. There are flaps at the top, which encapsulate the active site and the ligand within it.

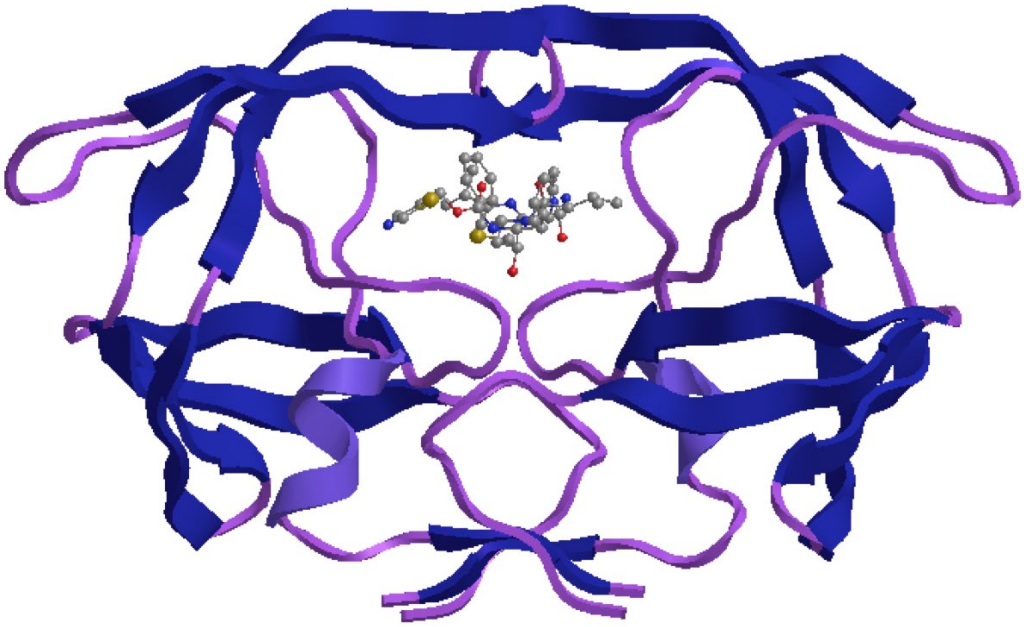


Figure 5 Crystal structure of ritonavir bound to the HIV protease enzyme.

3. Rotate the structure to view it from different angles.

In the following viewpoints (Fig. 6), the structure has been rotated 90° in the y-axis and 90 ° in the x-axis, showing that the inhibitor essentially traverses the width of the protein.

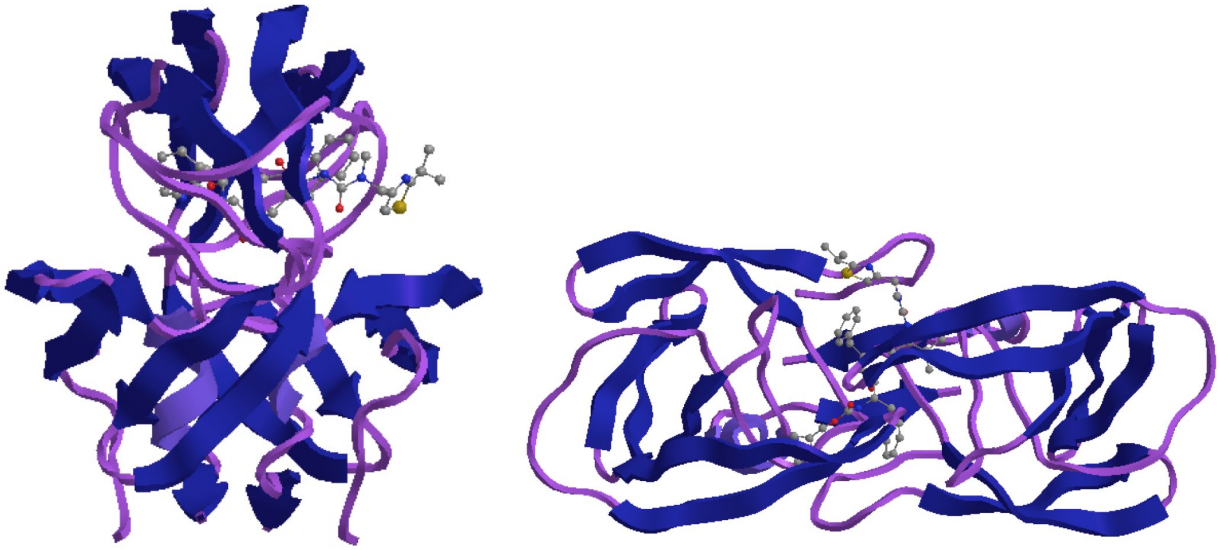
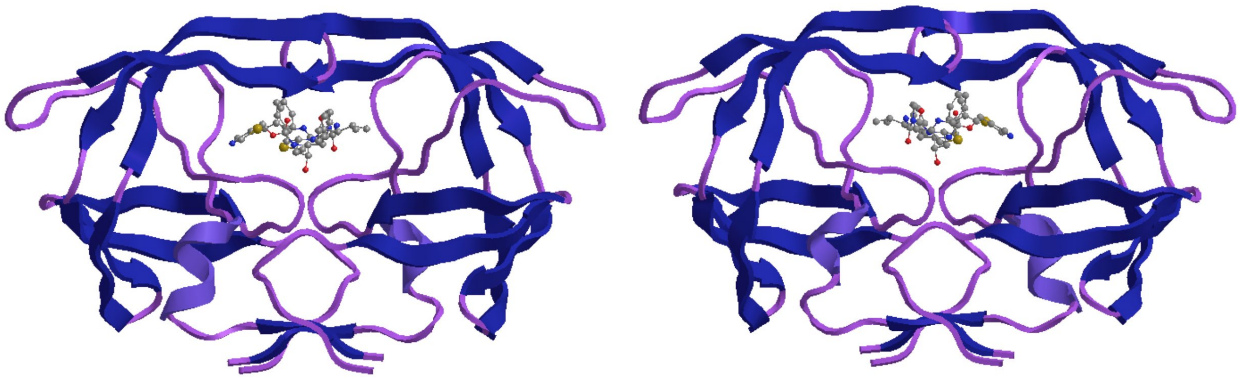


Figure 6 Different perspectives of the protein/ritonavir crystal structure.

If you return to the original viewpoint (Fig. 5) and rotate the structure 180 ^{o} in the y-axis, you will see that the same protein structure is represented (Fig. 7). This demonstrates the symmetry of the protein. You can tell that a rotation has taken place by looking closely at the ligand, which is not symmetrical.



A B

Figure 7 The protein/ritonavir complex viewed A) from the original viewpoint, and B) after 180^{o} rotation round the y-axis.

PART C Analysis of various components of the protein/ligand complex.

The **Model Explorer** table is useful in identifying different parts of the structure. There should be a tab for this at the left-hand margin of the window. Failing that, you can access it from the **View** menu by *choosing* **Model Explorer**. We will now identify the main components of the protein/ligand complex.

1. Open the Model Explorer table.

There are four entries for the protein/ligand complex- **Chain B, Chain A, Solvent** and **Backbone** (Fig. 8).

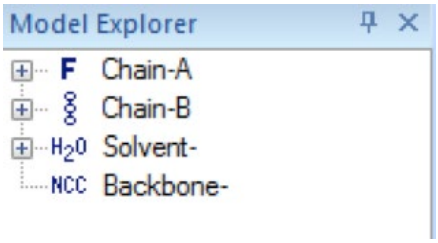
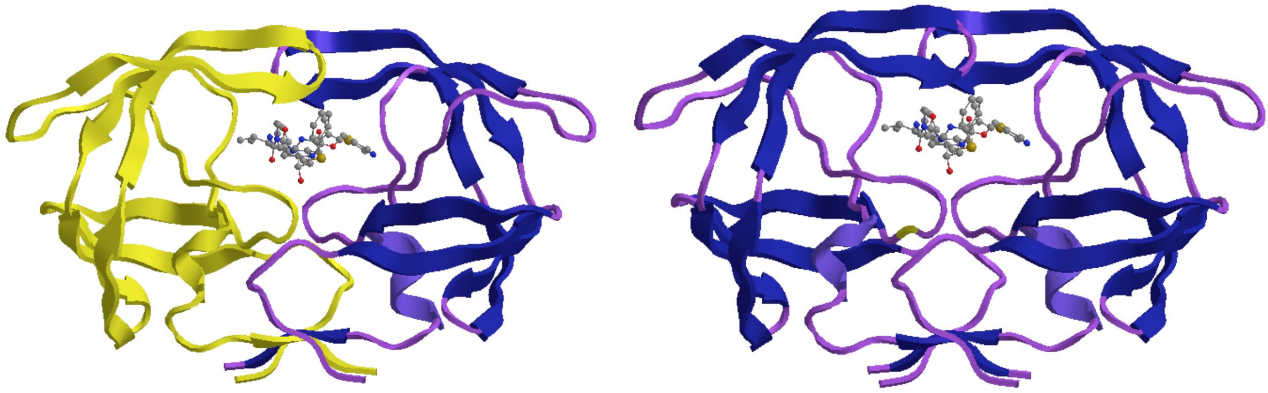


Figure 8 The Model Explorer table.

2 Identify the components of the protein-ligand complex

Click* on **Chain A and one of the proteins making up the protease enzyme will be highlighted in yellow (Fig. 9A).
Click* on the + sign to the left of **Chain A. This opens up a list of the constituent amino acids in that chain.
Click* on **Asp-25 and the position of that amino acid will be highlighted in yellow (Fig. 9B).
Click* on the – sign next to **Chain A to collapse this list.



A B

Figure 9 A) Chain A highlighted. B) Asp-25 in chain A highlighted.

Click* on **Chain B. The other protein subunit will now be highlighted along with ritonavir itself (Fig. 10A).
*Expand **Chain B** (Fig. 10D) and *click* on **Ligand-2**. Ritonavir is now highlighted (Fig. 10B).
Click* on **Fragment 1-99, and the protein subunit for Chain B is highlighted (Fig. 10C).

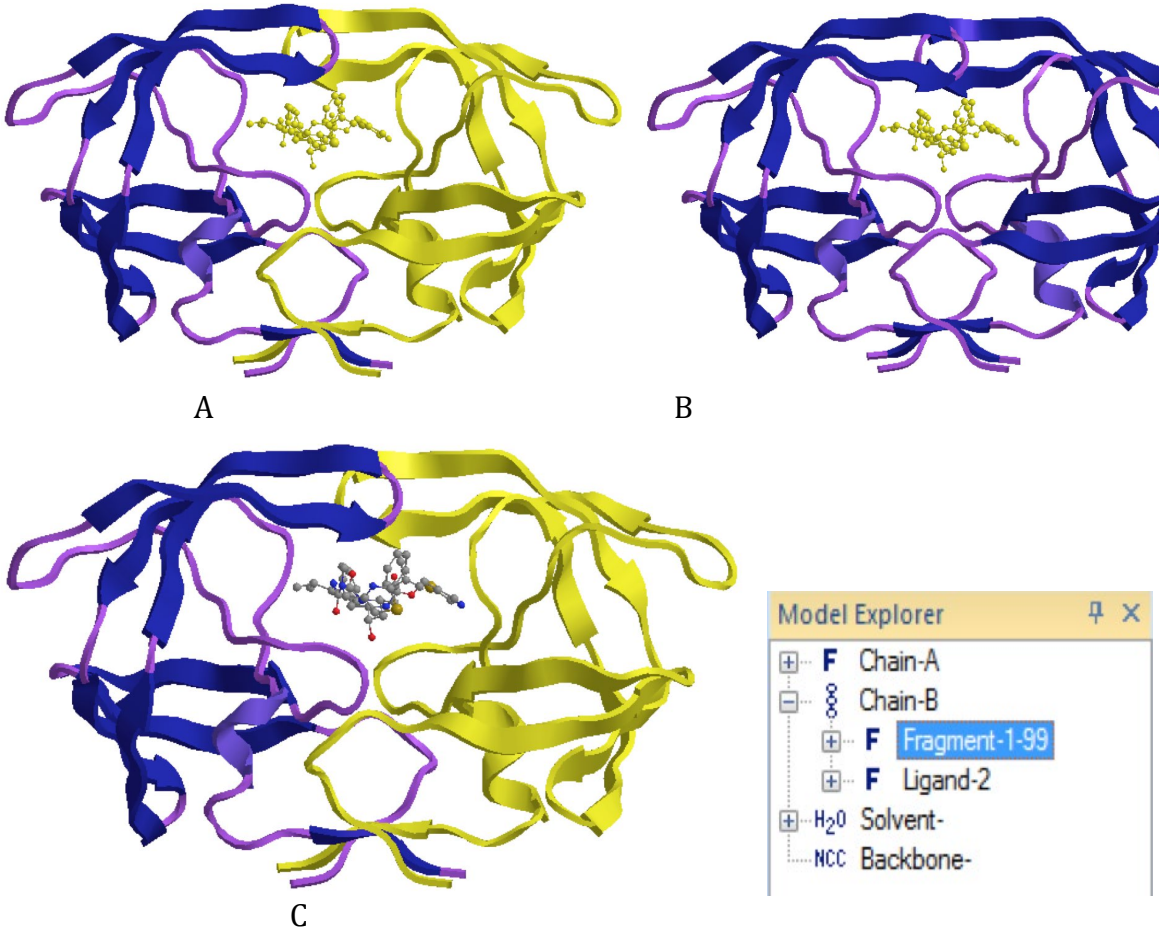


Figure 10 A) Ritonavir and Chain B highlighted, B) Ritonavir, C) Chain B, D) Model Explorer table of expanded Chain B.

PART D Analysis of ritonavir and the active site

In this part, we will look more closely at the active site and identify amino acids that are close to ritonavir. We will also identify the positions of the catalytic aspartate residues Asp-25 and Asp-25'.

1. Zoom into the binding site.

Choose* the **zoom tool (or scroll the mouse) to focus in on the binding pocket.
This allows you to study how ritonavir interacts with surrounding amino acids (Fig. 11).

2. Identify the amino acid residues close to ritonavir

*From the **View** menu, *choose* **Model Display**, then **Show Residue Labels**.
It is now possible to identify Asp 25 and Asp25'. They appear to be some distance from the hydroxyl group in ritonavir. However, the ribbon is only showing the peptide backbone, and does not show the side chains which stretch up to the ligand. We will see those side chains more clearly later on in the exercise. (see also Introduction to Medicinal Chemistry, 6th edition, section 20.7.4.4)

3. Highlight Asp-25 and Asp-25'

**Expand* chain A and Chain B in the Model Explorer table by *clicking* on the + signs to the left of the entries.
**Click* on the entry for Asp-25 in chain A to highlight it.
**Scroll* down to the entry for Asp-25 in chain B. With the control key depressed, *click* on that entry and both aspartate residues will be highlighted (Fig. 11).

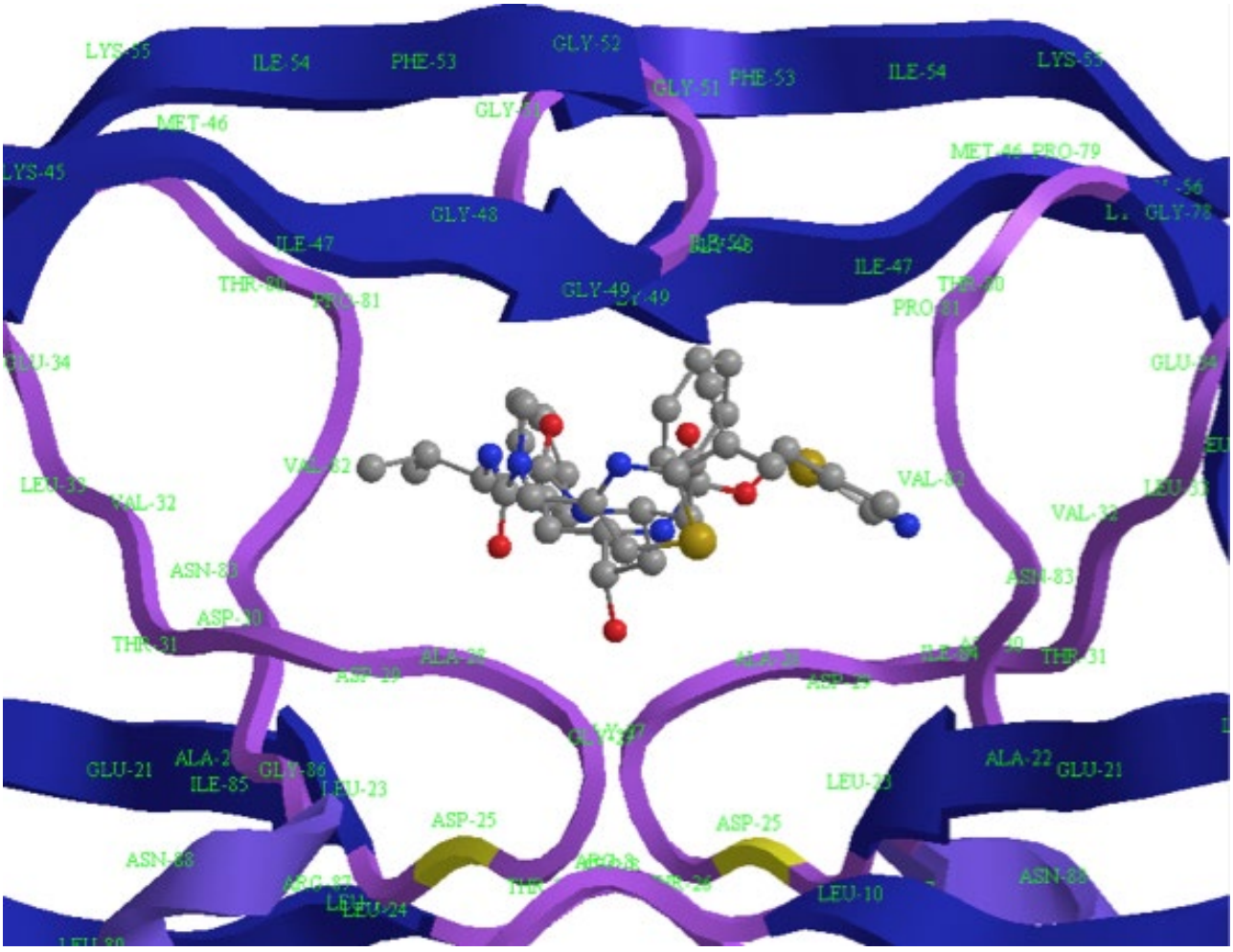


Figure 11 Ritonavir in the binding pocket of HIV protease with the catalytic aspartate residues highlighted.

PART E Identifying the active conformation of ritonavir.

In order to study the active conformation of ritonavir, we will extract the ligand from the active site and compare it with the energy-minimised conformation created in part A.

1. Extract ritonavir from the active site.

Choose* the **Select tool  , then *double click* on any part of ritonavir to select the whole molecule.

* From the **Edit** file, *choose* **Copy**.

**Open* a new Chem3D window and *paste* the structure into the new window.

A ball and sphere model for ritonavir will now be visible (Fig. 12). Do not energy minimise the structure or you will lose the active conformation (Note 1). Note that the structure is a fairly elongated conformation with substituents positioned alternately on each side of the main chain. This is typical of ligands that are designed to mimic the substrate of an enzyme. The substituents access the binding pockets (subsites) that are normally occupied by the side chains of protein substrates.

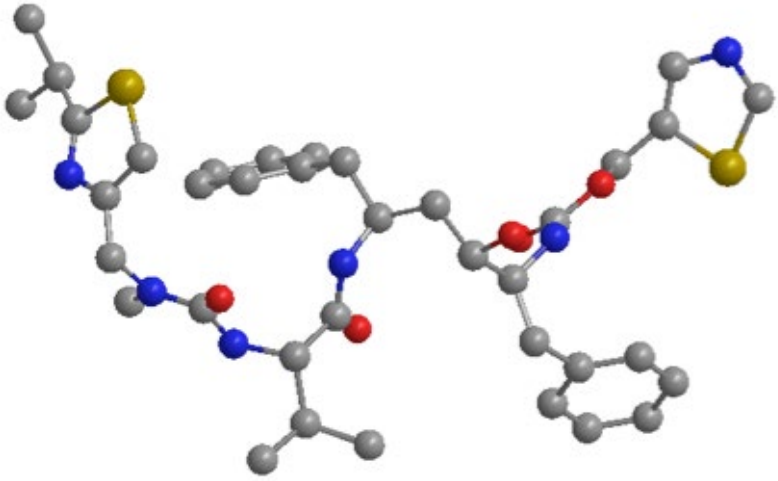


Figure 12 Active conformation of ritonavir.

Note 1 Energy minimisation is not valid since the true hybridisation state of the atoms has not been defined. For example, the aromatic carbons are treated as sp^3 hybridised if energy minimisation is carried out). It is also important to appreciate that there is a certain level of error involved in determining structures by X-ray crystallography, and that the bond angles and bond lengths are not at their optimal value in crystal structures.

2. Compare the active conformation of ritonavir with the energy-minimised structure of ritonavir from part A.

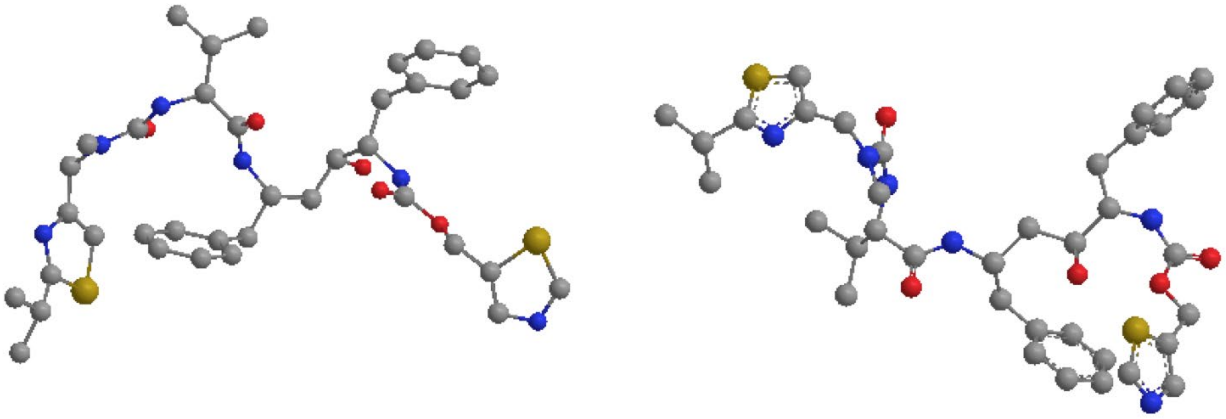
**Copy* the energy-minimised structure of ritonavir obtained in part A, and *paste* it into the same window as the active conformation.

*From the **View** menu, *choose* **Model Display**, then **Show Hydrogen Atoms**. Select **Hide**.

*From the **View** menu, *choose* **Model Display**, then **Show Lone Pairs**. Select **Hide**.

*Orientate the molecules such that they are roughly aligned. It is possible to *translate* or *rotate* one molecule without moving the other if it is first selected, and the translation/rotation is carried out with the shift key depressed.

A comparison of the structures shows that they are both extended conformations, but they are not the same (Fig. 13). The main chain is orientated differently as are the Rings and the side chains.



A B

Figure 13 Comparison of A) the active conformation of ritonavir with B) the energy-minimised conformation of ritonavir.

PART F Create a model binding site of the active site with ritonavir and labelled amino acids

The following process will allow you to extract ritonavir along with the amino acids closest to it in the active site. These amino acids are the ones most likely to be forming intermolecular bonds with the ligand.

1. Select ritonavir and the amino acid residues within 4Å of the structure.

**Zoom* in on the ligand in the binding site.
**Double click* on any of the atoms to select it.
* Keep the mouse cursor over the selection and *right click* the mouse to open a menu.
* *Choose* **Select**, then **Select groups within Distance of Selection**. *Choose* **4 Angstrom.**
Ritonavir and the closest amino acid residues will now be highlighted in the main window (Fig. 14)**.**

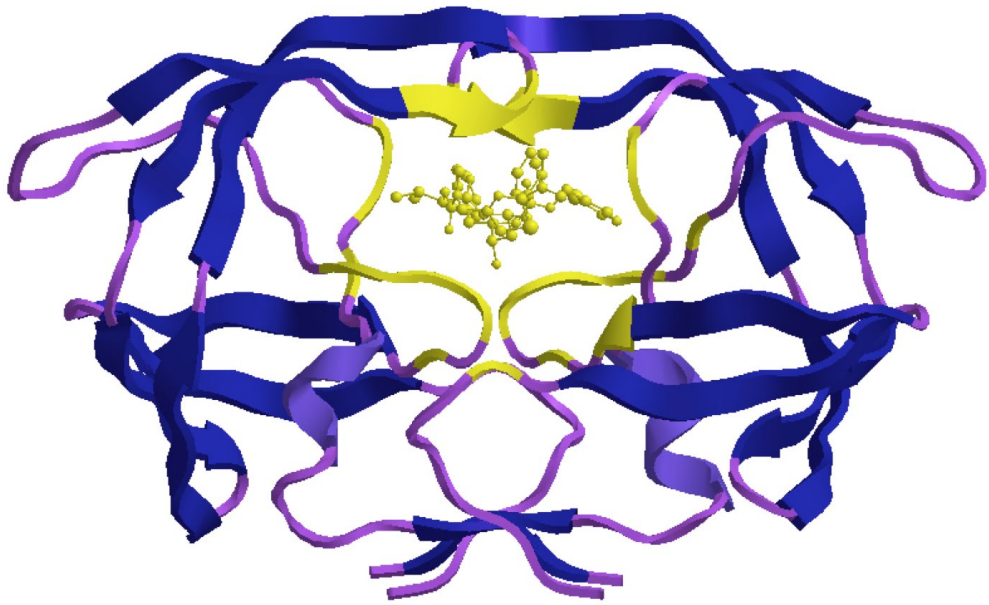


Figure 14 Selection of ritonavir and amino acid residues within 4Å.

2. Copy the ligand selection and paste it into a new window.

*From the **Edit** menu, *choose* **Copy**.
**Open* a new window and *paste* the selection into the new window (Fig. 15).

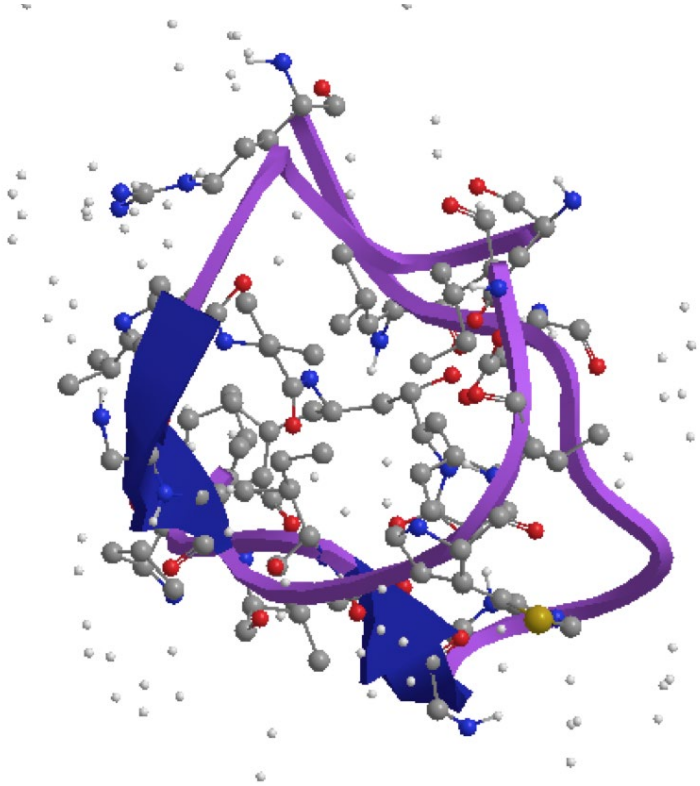


Figure 15 Initial pasted selection.

3. Modify the model to distinguish between ritonavir and the amino acid residues.
The initial paste is quite confusing looking and does not easily distinguish between ritonavir and the surrounding amino acid residues. Carry out the following steps to clean the model up.

*From the **View** menu *choose* **Model Display**, then **Display Mode.** *Select* **Sticks**.
*From the **View** menu, *choose* **Model Display** then *select* **Show Hydrogen Atoms.** Choose **Show Polar**.
*From the **View** menu, *choose* **Model Display** then *select* **Show Hydrogen Bonds.** *Choose* **Show Intermolecular**.
*In the **Model Explorer** table, *click* on **Solvent**, then *right click* the mouse to reveal a menu. *Choose* **Cut.** This simplifies the model and allows the structure to be rotated and translated more easily. Additional Solvent entries that appear as white spheres can be cut in the same way.
*To alter the ligand back to ball and stick, *select* the ligand by finding it in the **Model Explorer** Menu. The ligand is labeled **Ligand-2** and is part of the entry for Chain B. Alternatively, *double click* on any of the atoms belonging to the ligand. Make sure that the mouse is placed over the selection and r*ight click* the mouse to produce a menu. *Choose* **Display Mode**, then **Ball and stick.**
*If residue labels are not already visible, go into the **View** menu and choose **Model Display.** Select **Show Residue Labels.**

A ball and sphere model for the ligand will now be visible surrounded by labelled amino acids (Fig. 16). Hydrogen atoms attached to oxygen and nitrogen atoms are visible on the amino acid residues, but are not displayed on the ligand. Intermolecular hydrogen bonds between the amino acid residues and ritonavir are also visible, but only where atoms on ritonavir are acting as hydrogen bond acceptors. It is not possible to identify any hydrogen bonds where ritonavir provides the hydrogen bond donor since those hydrogen atoms are not shown.

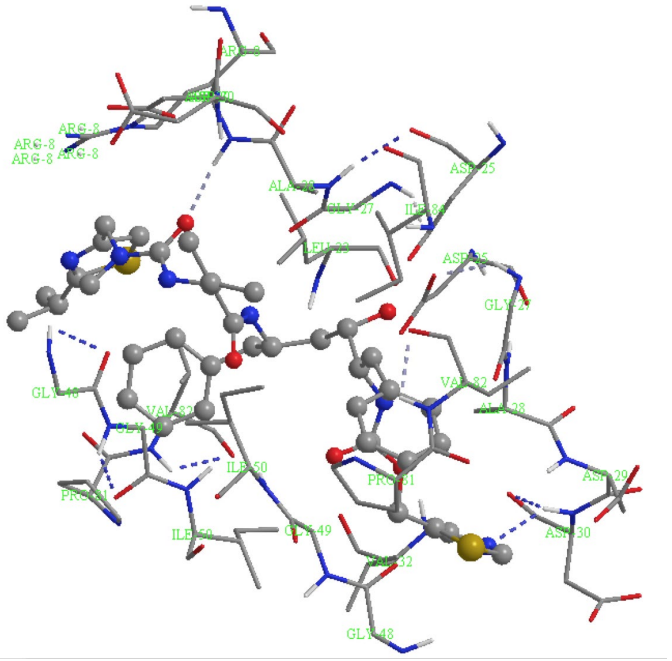


Figure 16 Ritonavir and labelled amino acid residues in the binding site

Specific H-bonds can be viewed close up using this model of the binding site. It helps to view the model in perspective to provide some depth of field to the image.

*From the **View** menu, *choose* **Model Display**, then **Perspective**.
The lengths of hydrogen bonds can be determined as follows.
Choose* the **Select tool.
*With the Shift key depressed, *click* on the atom acting as the HBD and the atom acting as the HBA.
*From the **Structure** menu, *choose* **Measurements**, then **Display Distance Measurement**.

4. Identify the H-bond between ritonavir and Asp-29.
Figure 17 shows a hydrogen bond measuring 2.1Å between the urea group of ritonavir and Asp-29. The carbonyl oxygen atom of the urea group is acting as the HBA, and the HBD is the NH proton of Asp-29. This is part of the peptide bond between Asp-29 and Ala-28.

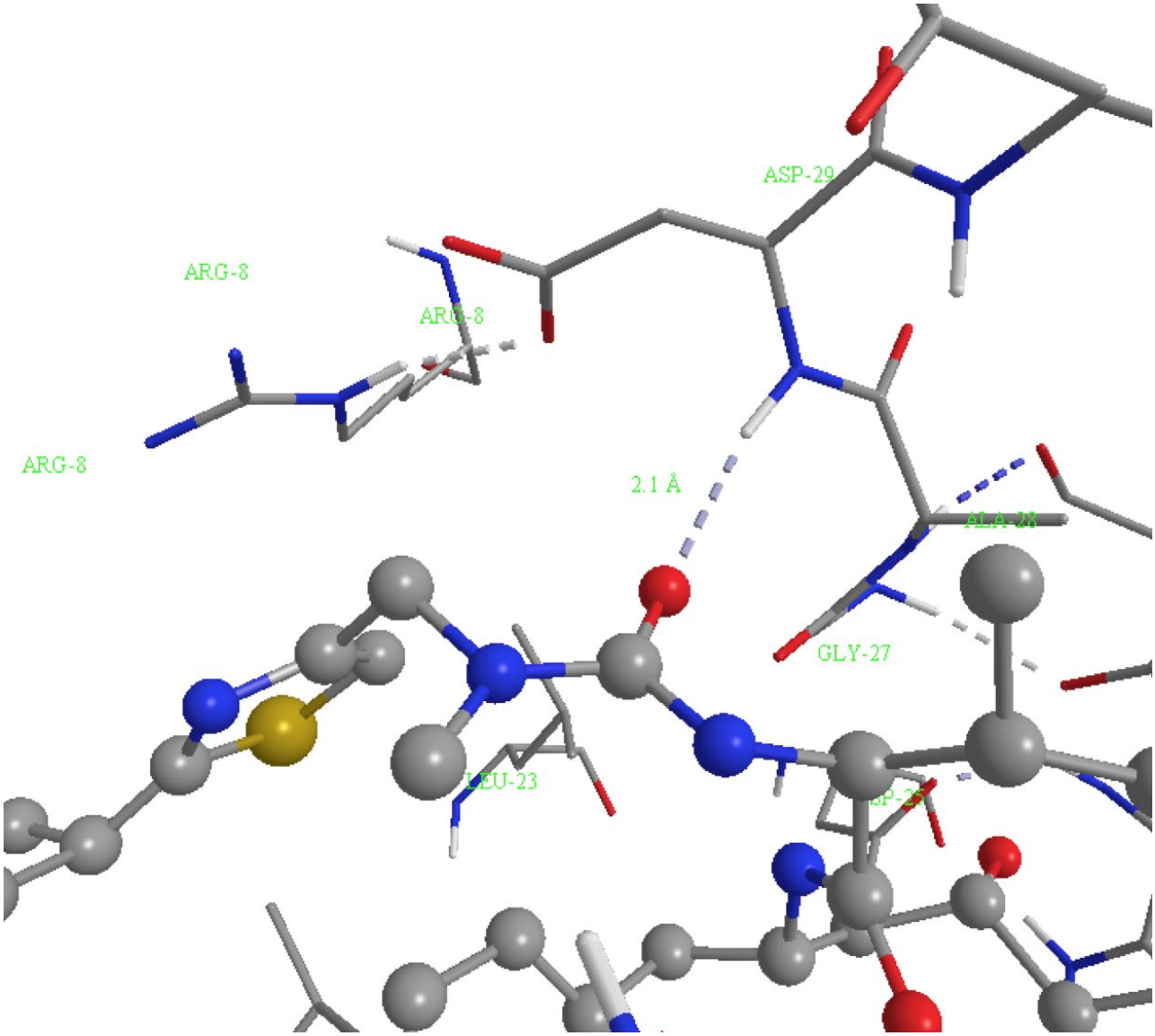


Figure 17 Hydrogen bond between ritonavir and Asp-29.

5. Identify the H-bond between ritonavir and Asp-30'.

There is a hydrogen bond measuring 2.2 Å between the unsubstituted thiazole ring of ritonavir and Asp-30' (Fig. 18). The nitrogen atom of the thiazole ring is acting as the hydrogen bond acceptor, and the hydrogen bond donor is the NH proton of Asp-30'. This is part of the peptide bond between Asp-29' and Asp-30'.

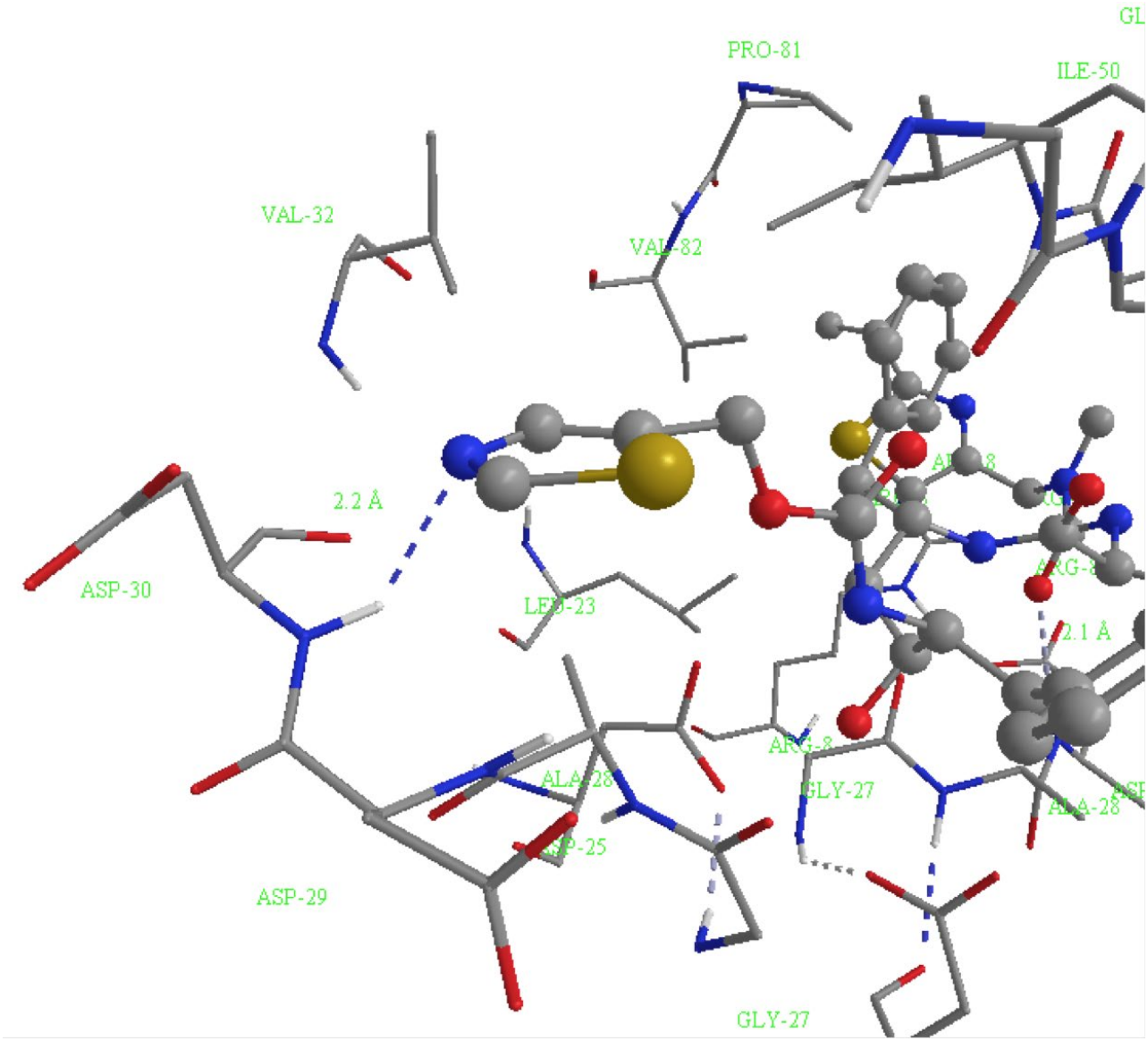


Figure 18 Hydrogen bond between ritonavir and Asp-30'.

5. Identify possible pi-pi interactions between ritonavir and Arg-8.

It is possible that pi-pi interactions are taking place between the substituted thiazole ring of ritonavir and Arg-8. This involves the thiazole ring interacting with the amidine group on the side chain of Arg-8 (Fig. 19). The atoms of the amidine group (N-C-N) are 3.5Å away from two carbons of the thiazole ring.

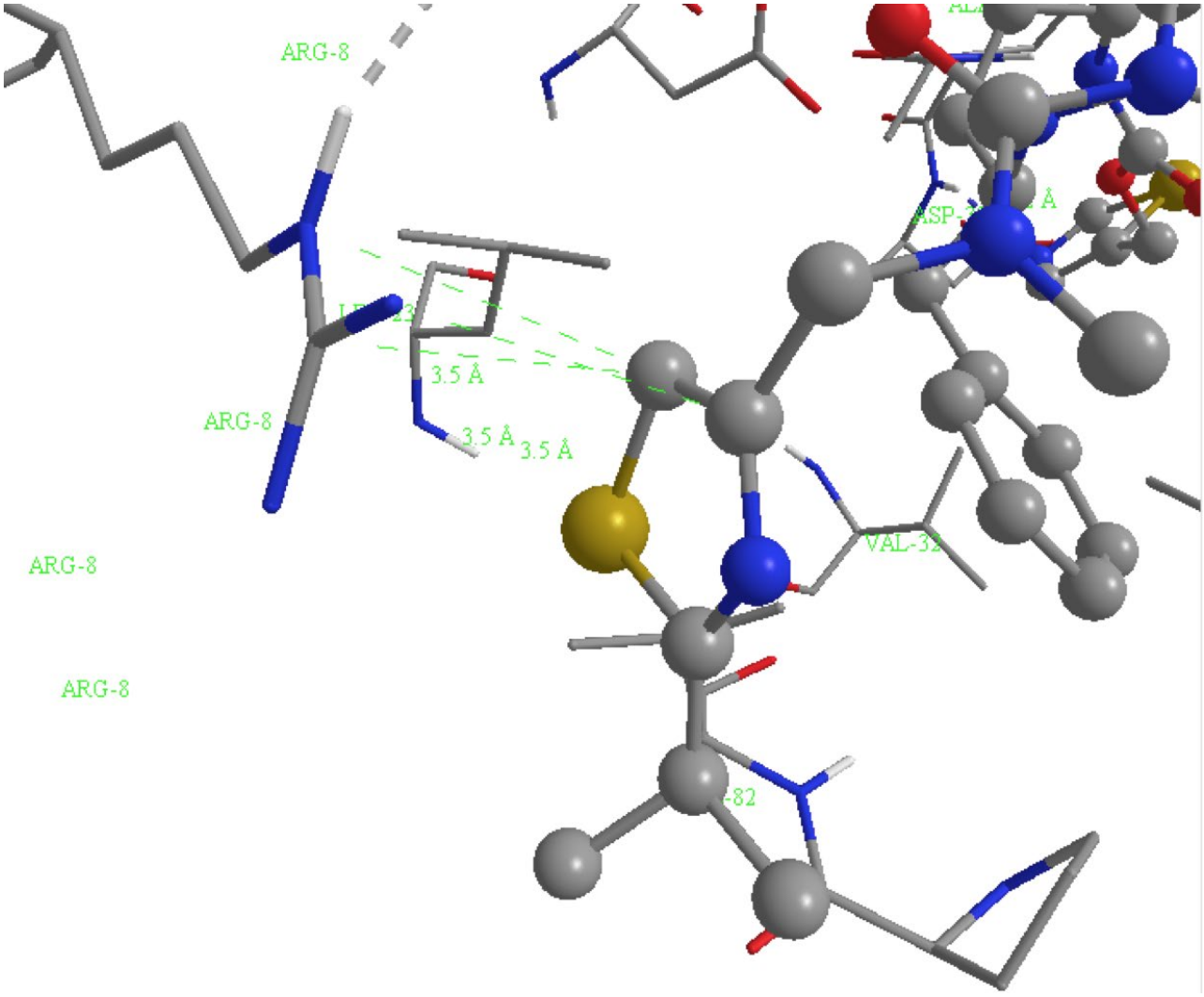


Figure 19 Possible pi-pi interactions with Arg-8.

PART G) Creating a model binding site – identification of HBDs on ritonavir.

The binding site created in part F allowed us to identify a couple of hydrogen bonds between ritonavir and surrounding amino acid residues. These involved groups on ritonavir that acted as hydrogen bond acceptors. However, it was not possible to identify any hydrogen bonds where groups on ritonavir acted as hydrogen bond donors, since no hydrogen atoms are shown on the ligand. We are now going to copy the ligand and closest amino acid residues in such a way that hydrogen atoms can be added to ritonavir. This will allow us to identify more hydrogen bonds, but it has to be appreciated that there are inaccuracies in the way hydrogen atoms are added to ritonavir.

1. Extract ritonavir along with the closest amino acid residues.

*Return to the window containing the imported pdb file of the crystal structure.
*The selection should still be highlighted in the window. If so, *choose* the **rotate** tool or **translate** tool and *click* in the main window. Do not use the **select** tool or you will deselect the highlighted region. If you need to redo the selection, do so as described in part F.
*From the **Edit** menu, *choose* **Copy As**, then *select* **ChemDraw Structure**.
*From the **File** menu, *choose* **New** to open a new window.
*From the **Edit** menu, *choose* **Paste**.
The pasted structure may look something like the following (Fig. 20).

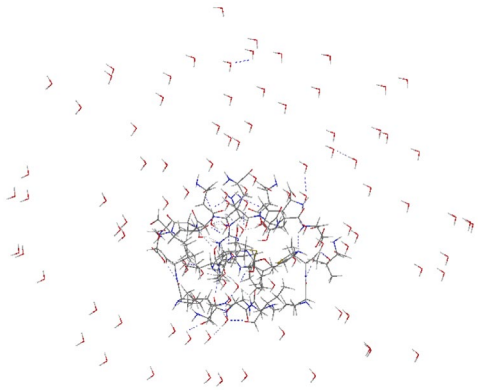


Figure 20 Pasted structure of extracted ligand and amino acid residues.

2. Modify the structure to allow a study of binding interactions

The model of the binding site needs to be tidied up, such that the ligand and the amino acid residues can be distinguished. The following series of operations will alter the model such that the amino acid residues are shown as sticks, while the ligand is shown as a ball and stick model. Only protons attached to electronegative atoms will be made visible and only intermolecular hydrogen bonds identified by the program will be displayed.

*From the **View** menu, *choose* **Model Display**, then **Display Mode**, then **Stick**. The ligand and the amino acids will now all be in the stick mode if they are not already so.
*From the **View** menu, *choose* **Model Display,** then **Show Hydrogen Atoms.** *Choose* **Show Polar**.
*From the **View** menu, *choose* **Model Display** then **Show Hydrogen Bonds** then **Show Intermolecular**.
*Go into the **Model Explorer** table and *select* the ligand (**Fragment-1**). Keep the mouse cursor over the selection and *right click* the mouse to open a menu. In the **Display Mode**, *choose* **Ball and Stick**.

*Use the **select** tool to *lasso* water molecules surrounding the binding site, then *choose* **Clear** from the **Edit** menu. It helps to rotate the model a number of times to identify all the surrounding water molecules. Leave the water molecules that are present within the binding site itself. Removing the surrounding water molecules allows the structure to be visualized more clearly and also allows rotation and translation to be carried out more easily.
*In the **Model Explorer** table, *click* on **Solvent**, then *right click* the mouse to reveal a menu.
Choose* **Visibility, then **Hide Group.**
The water molecules within the binding site are now hidden making visualization of interactions easier. They can be revealed at a later point to identify any water molecules acting as hydrogen bond bridges.
You should now have the following model of the binding site (Fig. 21). Ritonavir is present as the ball and stick model, surrounded by amino acid residues shown as stick models. The dotted lines show hydrogen bonds that have been identified by the program.

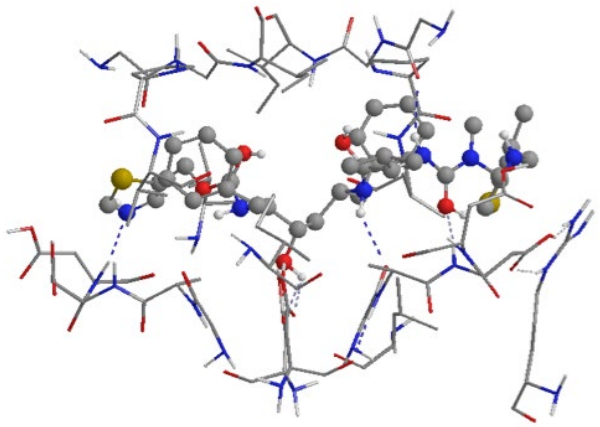


Figure 21 Ritonavir and closest amino acid residues.

3. Correct the ligand structure by removing extraneous hydrogen atoms.
Unfortunately, the addition of hydrogen atoms to the ligand is not accurate since the hybridisation of the atoms in the copied ligand structure has not been properly defined. This means that the program assumes that all the atoms are fully saturated sp^3 hybridised atoms. Therefore, it is necessary to hide hydrogen atoms that should not be present. These offending atoms are on the three carbonyl oxygens and the two nitrogen atoms within the heteroaromatic rings (Fig. 22A). These can be hidden manually by selecting the offending hydrogen atom, then *right clicking* the mouse as you hold the mouse cursor over the selected atom. From the menu that appears, *choose* **Visibility**, then **Hide Atom**.

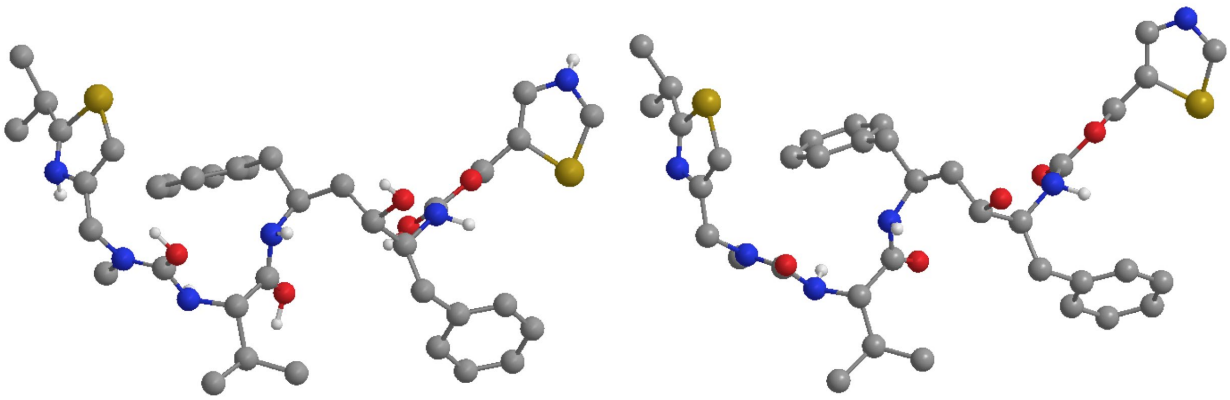


Figure 22 A) Structure containing erroneous hydrogen atoms. B) Corrected structure.

Note: Although the structure has been corrected to hide hydrogen atoms that should not be present, the orientation of some of the remaining hydrogen atoms may be faulty. For example the NH protons of amide bonds are not orientated properly since they should be in the plane of the amide group. Instead, they are orientated as if the nitrogen atom is sp^3 hybridised.

4. Identify hydrogen bonds between ritonavir and the aspartate residues Asp-25 and Asp-25'

*From the **View** menu, *choose* **Model Display**, then **Perspective**.
**Zoom* into the region containing Asp-25 and Asp-25'
* Measure the distances between the hydroxyl group of the transition state isostere of ritonavir and the CO_2H groups of Asp-25 residues (Fig. 23).

You should find four possible interactions measuring 1.8Å, 2.2Å, 2.2Å and 2.3Å forming a symmetrical hydrogen bonding network where the oxygen of the alcohol group interacts with the acidic proton of each CO_2H group, while the alcohol proton interacts with the carbonyl oxygen of each COOH group.

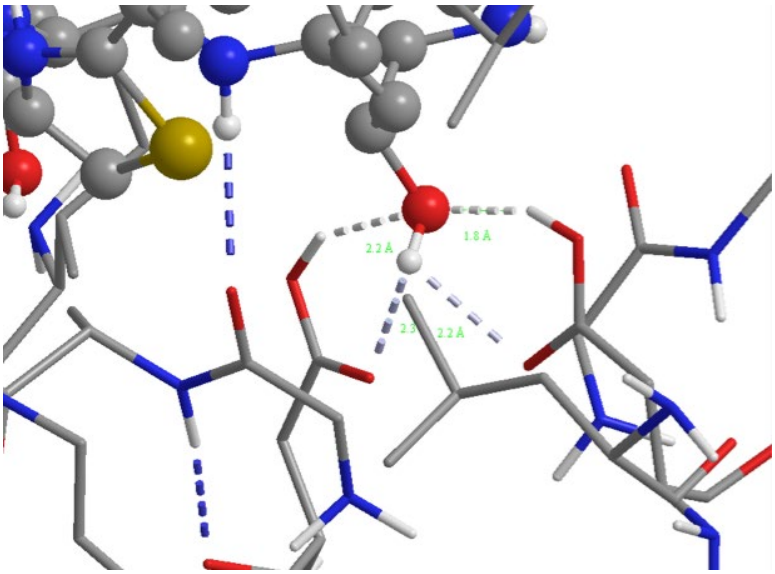


Figure 23 Distances between the transition-state isostere and aspartate residues (compare figure 24).

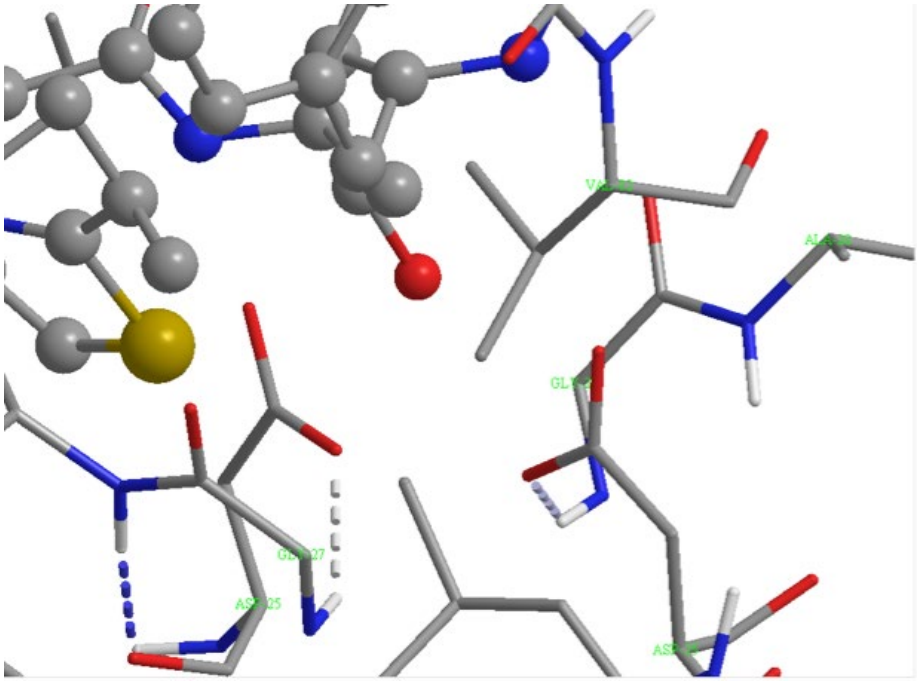


Figure 24 Identification of Asp-25 and Asp-25' (compare figure 23).

5. Identify the other hydrogen bonds that are present and measure their lengths.

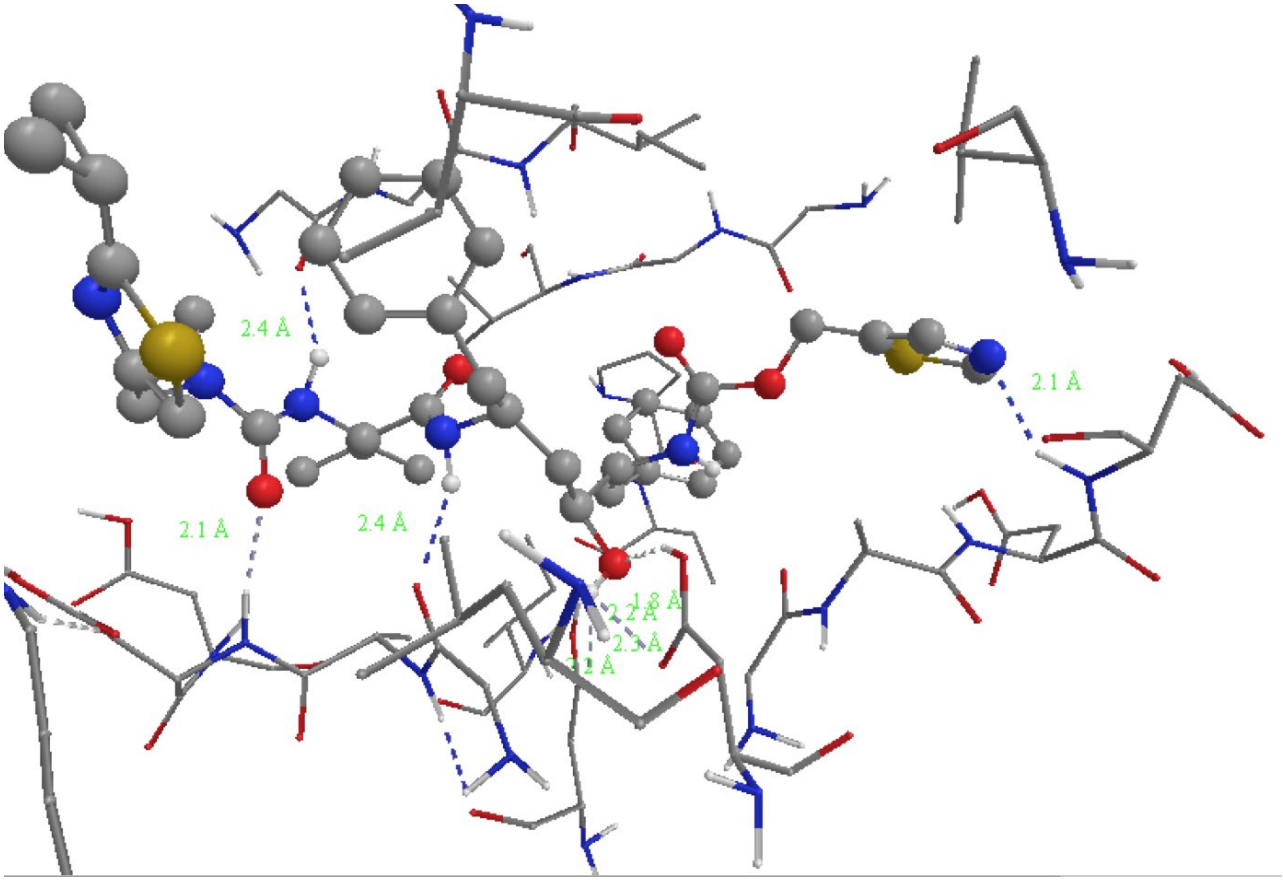


Figure 25 Other hydrogen bonds between the ligand and the binding site.

Apart from the hydrogen bonds involving the transition-state isostere, there are four other hydrogen bonds visible in figure 25. These are as follows from left to right (see also figure 26).

*Between the urea group of ritonavir and Asp-29 (see also figure 17),
*Between the urea NH proton of the ligand and the amide carbonyl of Gly48 (length 2.4Å). This peptide bond is between Gly-48 and Gly-49.
*Between the amide NH of the ligand with the amide carbonyl oxygen of Gly27 (length 2.4Å). This peptide bond is between Gly-27 and Ala-28.
*Between the unsubstituted thiazole ring of ritonavir and Asp-30' (see also figure 18).

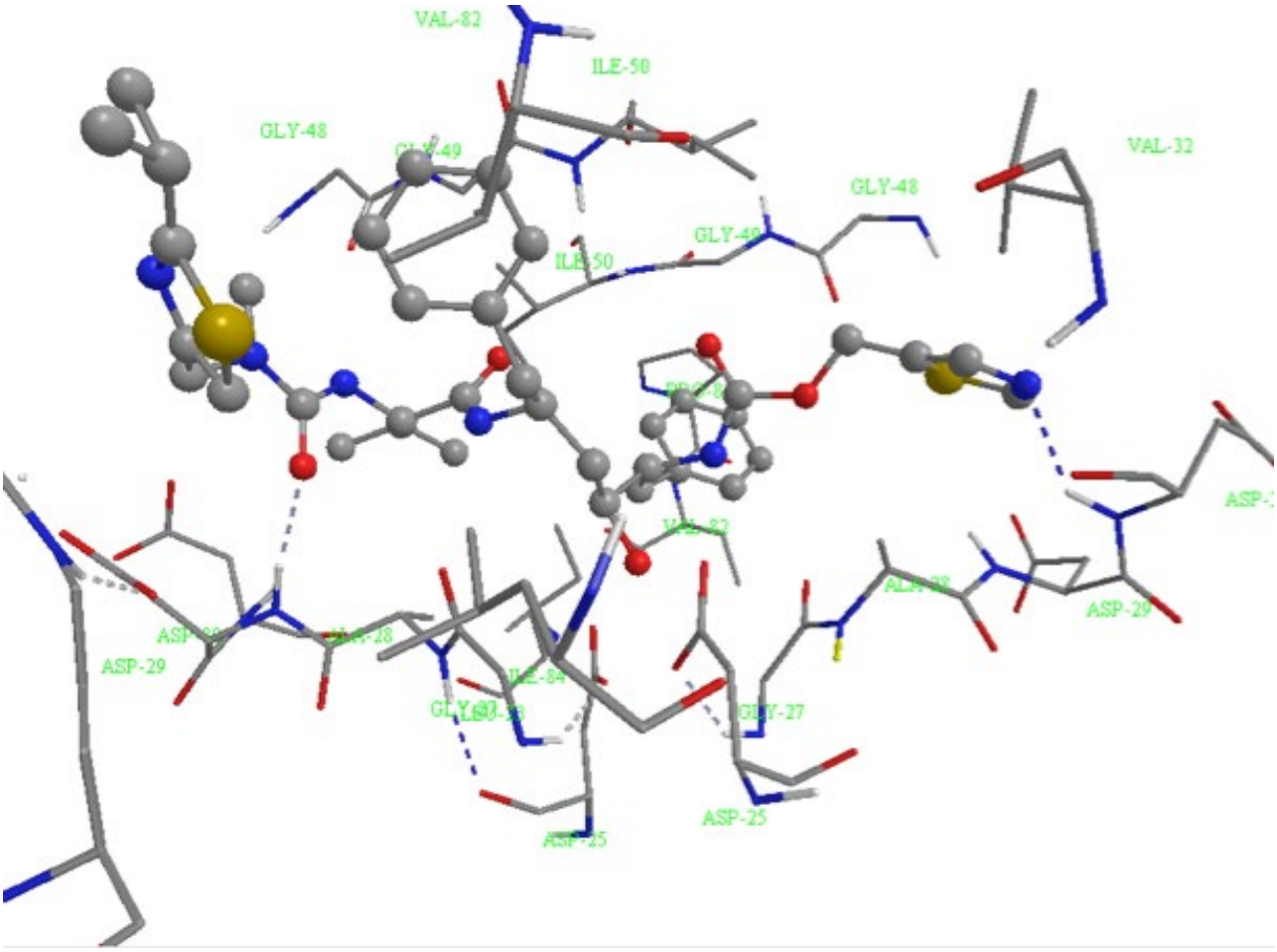


Figure 26 Identification of amino acid residues shown in figure 25.

6. Identify the interaction between ritonavir and the flap region
The interaction of ritonavir with the flap region involves a bridging water molecule. Therefore, it is necessary to reveal the water molecules within the active site.
*Go into the **Model Explorer** table.
Select* **Solvent. Keep the mouse cursor within the **Model Explorer** table and *right click* the mouse.
Choose* **Visibility, then **Show Group**.
**Zoom* into the relevant area.
The orientation of the water molecule may differ from what is shown in figure 27. In figure 27, there are two hydrogen bonds shown, but other hydrogen bonds are possible depending on the orientation of the water molecule. As far as ritonavir is concerned, the carbonyl oxygen

of the amide group can act as an HBA in the interaction with the water molecule. The water molecule can then hydrogen bond to the NH groups of Ile-50 and Ile-50'. The NH groups are part of peptide bonds between Ile-50 and Gly-49 and between Ile-50' and Gly-49'.

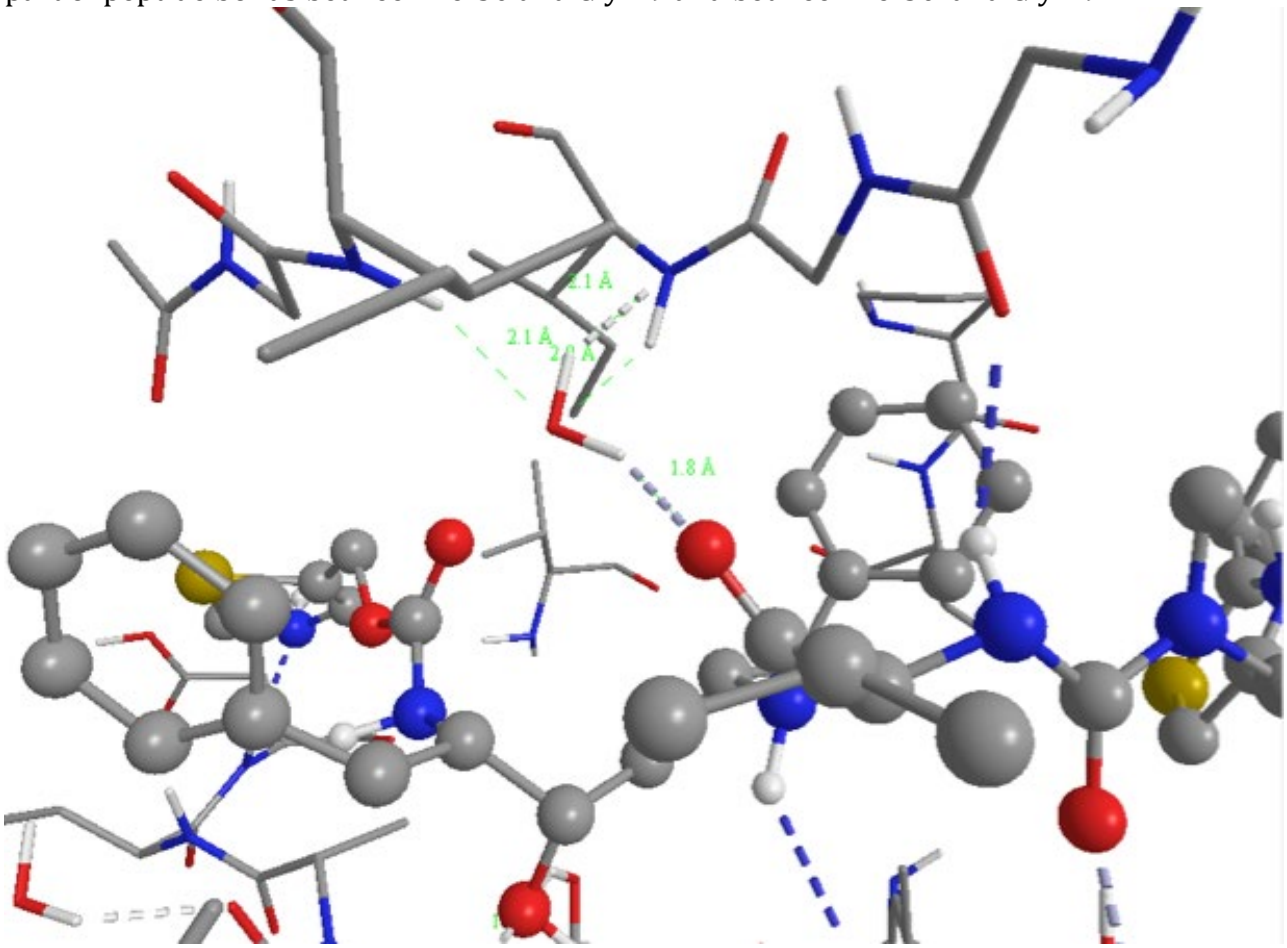


Figure 27 Hydrogen bonding to Ile-50 and Ile-50' via a bridging water molecule.

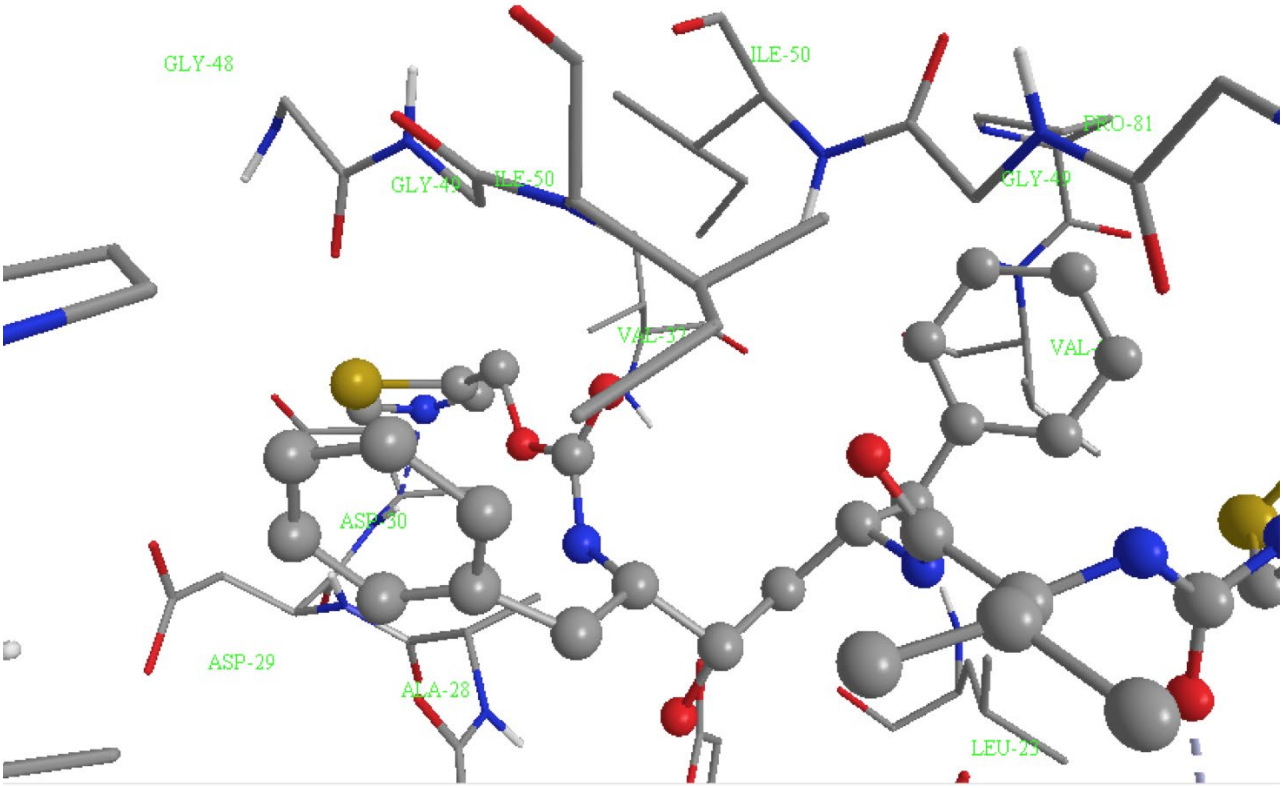


Figure 28 Identification of amino acid residues shown in figure 27.

PART H The PoseView image

Useful information can be obtained about how a ligand binds to a binding site from the protein data bank web site. The protein data bank can be accessed at www.rcsb.org.
*In the search box provided on the home page, enter the PDB code required (1hxw), then *click* on **Go**. This takes you to the front page of that file.
Scroll* down the page to a section headed **Small Molecules, which refers to any ligands bound to the protein.
*Under the section on ligands, there are two diagrams shown under **2D Diagram and Interactions**. The left hand structure shows the structure of the ligand. The right hand diagram shows intermolecular binding interactions between the ligand and key amino acids within the binding site.
**Click* on the second diagram to get a window showing an expanded view of a Poseview Image of binding interactions (Fig. 29). Hydrogen bonds are shown by dashed lines. Hydrophobic pockets are shown by green lines with the amino acids lining the pockets identified.

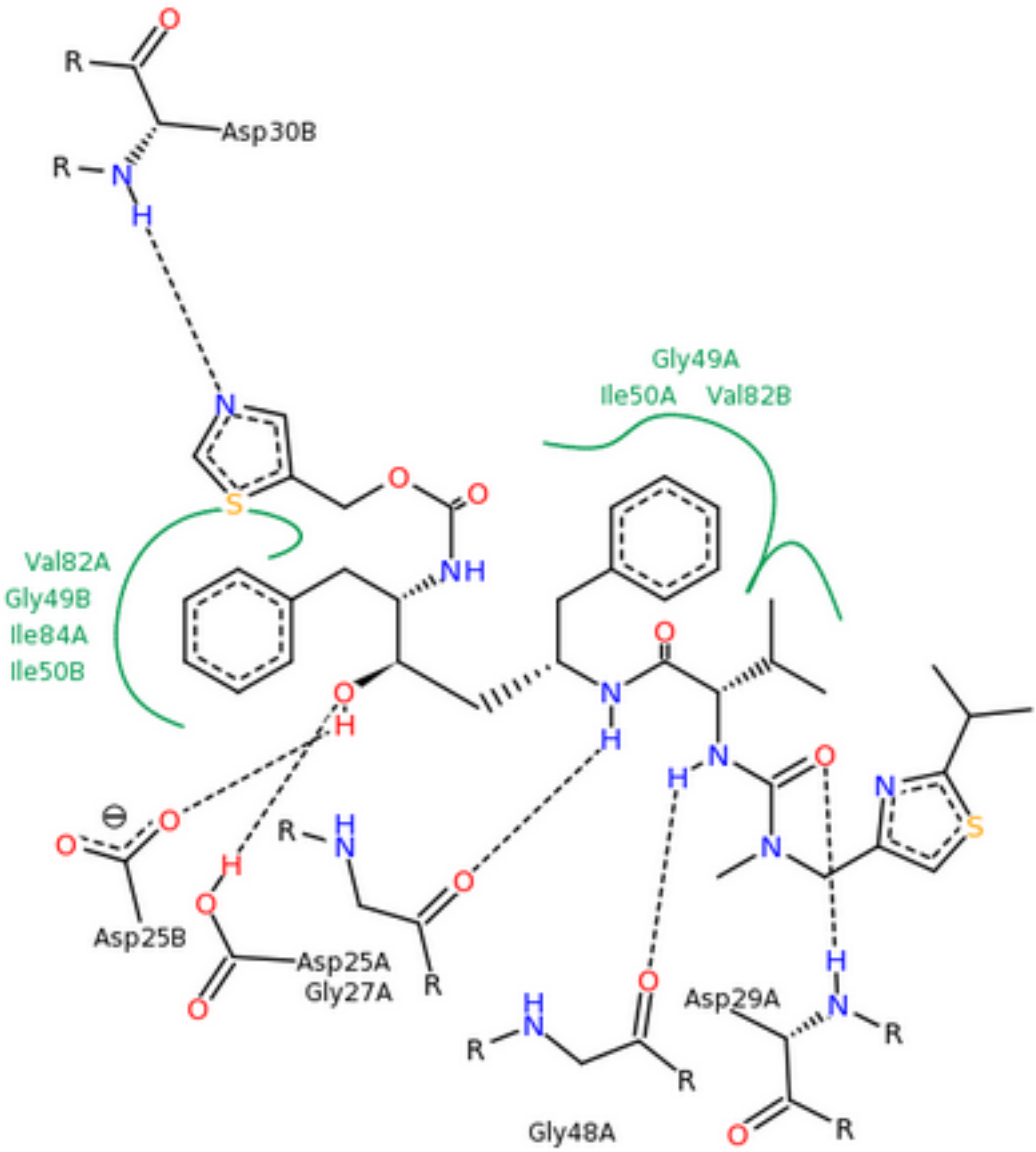


Figure 29 PoseView image of the ligand and identified binding interactions.