Journal of Computer-Aided Molecular Design, **14:** 531–544, 2000.
KLUWER/ESCOM

Computer-aided design and activity prediction of leucine aminopeptidase inhibitors

J. Grembecka[a], W.A. Sokalski[b] & P. Kafarski[a]
[a]*Institute of Organic Chemistry, Biochemistry and Biotechnology;* [b]*Institute of Physical and Theoretical Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland (Tel.: (+48)(+71)3202137; Fax: (+48)(+71)3284064; E-mail jola@neon.ch.pwr.wroc.pl)*

Received 19 April 1999; Accepted 17 January 2000

Key words: activity prediction, drug design, leucine aminopeptidase (LAP), ligand-receptor interactions, LUDI, phosphonic acid inhibitors

Summary

The Ligand Design (LUDI) approach has been used in order to design leucine aminopeptidase inhibitors, predict their activity and analyze their interactions with the enzyme. The investigation was based on the crystal structure of bovine lens leucine aminopeptidase (LAP) complexed with its inhibitor – the phosphonic acid analogue of leucine (LeuP). More than 50 potential leucine aminopeptidase inhibitors have been obtained, including the most potent aminophosphonic LAP inhibitors with experimentally known activity, which have been the subject of more detailed studies. A reasonable agreement between theoretical and experimental activities has been obtained for most of the studied inhibitors. Our results confirm that LUDI is a powerful tool for the design of enzyme inhibitors as well as in the prediction of their activity. In addition, for inhibitor-active site interactions dominated by the electrostatic effects it is possible to improve binding energy estimates by using a more accurate description of inhibitor charge distribution.

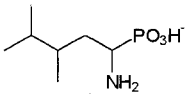
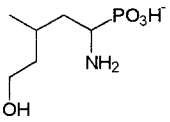
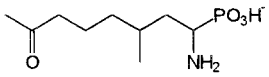
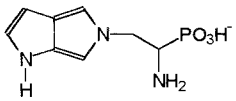
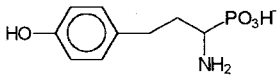
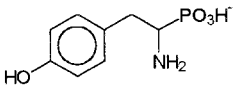
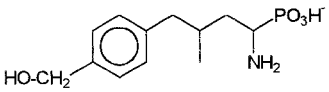
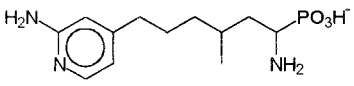
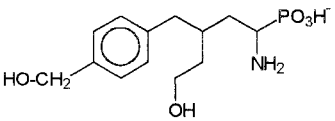
Introduction

Leucine aminopeptidase [LAP, α-aminoacyl-peptide hydrolase (cytosol) E.C.3.4.11.1] is an exopeptidase that catalyzes the removal of amino acids from the N-terminus of a peptide or protein [1]. Similar to other aminopeptidases, this enzyme is of significant biological and medical importance because of its key role in protein modification and degradation as well as in the metabolism of biologically active peptides [2, 3]. Altered activity of leucine aminopeptidase has been associated with several pathological disorders, such as cancer and eye lens cataracts [4]. Moreover, LAP may play an important role in the early stages of HIV infection [5] and thus serum activity of this enzyme may be useful as a surrogate marker for HIV infection and as a response to chemotherapy.

Bovine lens leucine aminopeptidase is a hexameric enzyme. Each of its six identical subunits exhibits independent catalytic activity and contains three zinc ions. Two of them are placed in the active site of the enzyme [1]. These metal ions are essential for catalytic activity because both participate in substrate binding and activation, including a possible role in the activation of the nucleophile. Leucine aminopeptidase exhibits substrate specificity towards L-enantiomers of N-terminal amino acids.

A number of various reactant analogue inhibitors have been reported to bind to LAP. Among these amino acid analogues: L-leucinal (K_i = 60 nM) [6], the phosphonic acid analogue of L-leucine (LeuP, K_i = 0.23 μM) [7], and the boronic acid analogue of L-leucine (K_i = 0.13 μM [8], all the inhibition constants are given for porcine kidney LAP) play a major role in leucine aminopeptidase inhibition. All of them are considered as transition state analogues and bind in a similar way to the LAP active site, which has been confirmed by the X-ray crystal structures of leucine aminopeptidase with L-leucinal and LeuP [9] (Figure 1).

Table 1. The structures, calculated activities and score values for new designed LAP inhibitors optimized by a CFF97 force field approach (the number in the inhibitor label indicates the Score_all value)

Inhibitors	Score_all values	Hydrogen Bond Score values	Lipo_Score values	Zinc_Score values	Calculated K_i values (μM)
 **Inh686**	686	197	228	458	0.14
 **Inh692**	692	277	180	457	0.12
 **Inh693**	693	287	219	460	0.12
 **Inh742**	742	291	166	457	0.038
 **Inh761**	761	283	216	459	0.024
 **Inh780**	780	300	192	460	0.015
 **Inh785**	785	305	269	459	0.014
 **Inh794**	794	338	272	457	0.011
 **Inh856**	856	384	314	456	0.002

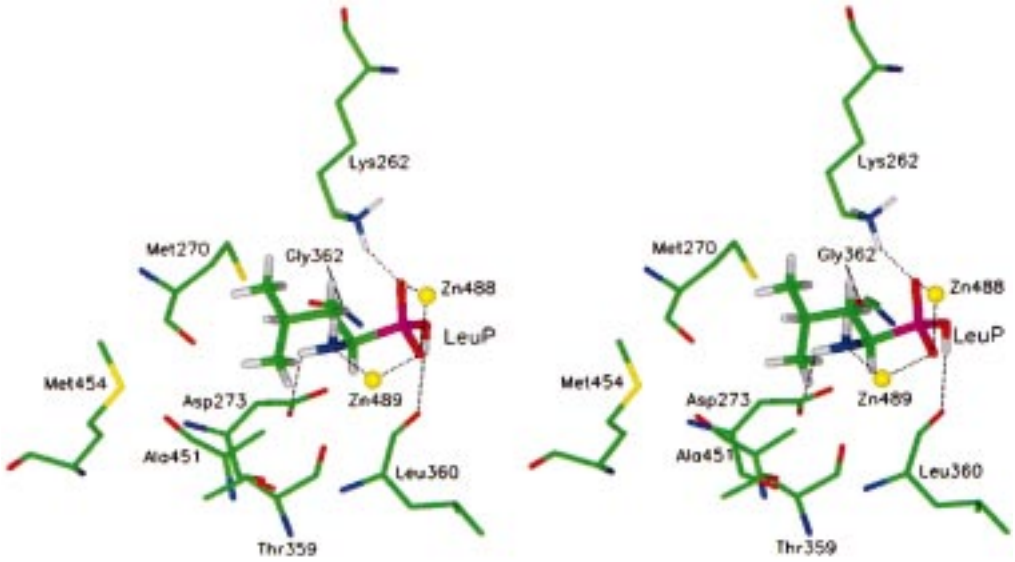


Figure 1. The binding mode of LeuP inhibitor by LAP; the coordinates of the complex were taken from the PDB (entry 1lcp).

The phosphorus analogues of amino acids and peptides are important compounds that mimick the tetrahedral *gem*-diolate transition state of peptide hydrolysis by many zinc peptidases [1]. Due to the interactions of their phosphonic group with zinc ions (Figure 1), these compounds are potent inhibitors of LAP and other zinc peptidases (e.g. thermolysin and carboxypeptidase A) and may exhibit activity in the femtomolar range [10, 11]. Among the phosphonic compounds, the strongest LAP inhibitors are analogues of natural amino acids: the phosphonic analogues of L-leucine ($K_i = 0.23$ μM) and L-phenylalanine ($K_i = 0.42$ μM, the K_i values with respect to porcine kidney leucine aminopeptidase) [7]. Thus, the rational design of the new phosphonic inhibitors of the enzyme, based on the LAP crystal structure, may result in its new, stronger, low molecular regulators. Moreover, the activity prediction of designed inhibitors by using theoretical methods may facilitate the selection of the most promising compounds for synthesis and activity determination.

The accurate prediction of binding affinity is one of the most important tasks in de novo ligand design to direct the synthesis of potential modeled ligands bound to receptors. One of the most promising methods for designing enzyme inhibitors and predicting their activity, implemented in the LUDI program, was proposed by Böhm and has been used to generate several new sets of active compounds [12–17]. In this paper we have applied this approach in order to generate LAP inhibitors and to analyze the nature of their activity. Thus, the main purpose of our work consists of:

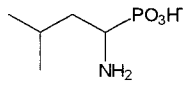
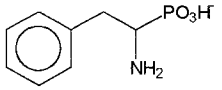
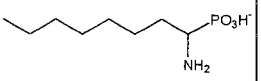
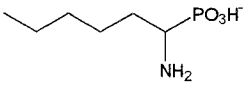
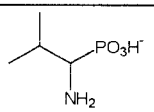
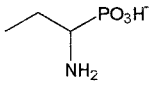
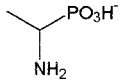
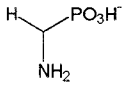
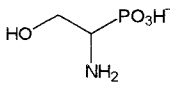
- the generation of leucine aminopeptidase inhibitors, with the focus on the enzyme pocket, which binds the hydrophobic chain of LeuP (the S1 pocket, Figure 1);
- the determination of the size and nature of the interactions in this pocket;
- the prediction of the activities of known leucine aminopeptidase inhibitors and their comparison with experimentally determined values;
- the analysis of the interactions of LeuP analogues with the active site of the enzyme.

Methods

The coordinates of leucine aminopeptidase and LeuP were obtained from the refined 1.65 Å X-ray structure of bovine lens leucine aminopeptidase complexed with the phosphonic analogue of leucine [1] (1lcp refcode in Brookhaven Protein Data Bank). The hydrogen atoms were added using the Insight 97.0 (MSI) program [18]. The protonation states of the residues were selected for pH=7.0, excluding Lys250, which is neutral, because the nitrogen atom electron lone pair in the side chain of this residue is coordinated by Zn489 [1].

The computer program LUDI [17] was applied to design LAP inhibitors using Silicon Graphics O2,

Table 2. The comparison of the score values for aminophosphonic acids in the conformation generated by LUDI as well as optimized by a CFF97 force field approach together with their activity: experimentally measured and calculated

Inhibitors	Computational methods	Score_all values	Hydrogen Bond Score values	Lipo_Score values	Zinc_Score values	Calculated K_i values (μM)	Measured K_i values (μM)
 **1 (LeuP)**	LUDI	678	234	175	450	0.208	0.23 (L)
	CFF97	668	175	213	452		
 **2 (PheP)**	LUDI	713	234	201	452	0.067	0.42 (L)
	CFF97	717	194	243	452		
 **3**	LUDI	636	234	225	450	0.407	0.47 (DL)
	CFF97	639	183	278	451		
 **4**	LUDI	648	234	186	450	0.311	1 (DL)
	CFF97	648	183	231	452		
 **5 (ValP)**	LUDI	679	233	142	450	0.338	1.2 (DL)
	CFF97	647	184	157	452		
 **6**	LUDI	644	234	106	450	0.501	3.6 (DL)
	CFF97	630	191	133	452		
 **7 (AlaP)**	LUDI	622	234	59	451	1.096	240 (DL)
	CFF97	596	183	83	456		
 **8 (GlyP)**	LUDI	600	233	38	450	2.818	1040
	CFF97	555	188	35	453		
 **9 (SerP)**	LUDI	592	242	67	451	4.786	1580 (DL)
	CFF97	532	171	56	451		

Onyx and Indy workstations. A systematic search of LUDI's fragment library, containing about 1000 structural fragments, was performed. The aminophosphonic fragment of LeuP was fixed and the Link mode of the program was used to generate new LAP inhibitors. The structures of designed inhibitors were obtained by the replacement of the hydrogen atoms or a chosen alkyl group of LeuP (replaced previously by hydrogen atom), by the structural fragments generated using LUDI. They are bound in the S1 pocket of the enzyme. More than 50 inhibitor structures were obtained in this manner. The values of the most important LUDI parameters used during the generation of new LAP inhibitors were as follows: Min Separation = 3; Link, Lipo and H-Bond Weights were set to 1.0; Aliphatic_Aromatic and Reject Bifurcated parameters were turned off; No_Unpaired_Polar and Electrostatic_Check parameters were turned on; Es Dist = 2.5; Max RMS = 0.5; Radius of Search was changed from 5 Å to 10 Å [17].

For a more detailed analysis, we selected six LAP inhibitors with known activity [7, 19], which were reproduced using LUDI (compounds **1–6** in Table 2). The L-PheP structure was obtained by removing the hydroxyl group from the L-TyrP structure (phosphonic analogue of L-tyrosine), a compound which was generated using the program, but its activity towards the enzyme has not been measured yet. Additionally, we added three other known leucine aminopeptidase inhibitors (compounds **7–9** in Table 2), which were not found in this way, probably due to their significantly lower activity towards LAP. They were included in the analysis for comparison. Two of them (AlaP and GlyP) were built by reduction of the hydrophobic fragment of the LeuP structure in the conformation generated by LUDI. The SerP structure was obtained by replacement of one of the hydrogen atoms in AlaP by the hydroxyl moiety.

We performed the optimization of each inhibitor placed in the active site of the enzyme with a CFF97 force field [20] using the Insight 97.0/Discover program [21]. The optimization process was performed for the system containing about 17,000 atoms, involving the protein molecule, the inhibitor molecule, 930 crystal water molecules and the additional 2566 water molecules, forming a 6 Å layer around the protein. The latter ones were added in the Insight II program, using the standard procedure. During the minimization process the system's degrees of freedom were gradually released, according to the stepwise procedure, starting from free hydrogen atoms (excluding the inhibitor molecule) and relaxing the remaining parts of the system in the following order: water molecules and the side chains of the enzyme, the main chain of LAP, the zinc ions and finally gradually relaxing the inhibitor structure. The distance constraints for all contacts of the zinc active site ions with their ligands (the enzyme residues and aminophosphonate group of the inhibitors [1]) were taken from the X-ray structure of the complex: LAP-LeuP and a force constant value of 500 kcal/mol$*$Å^2 were applied in all steps of the energy minimization procedure. This allowed us to maintain a similar location of the aminophosphonic fragments and other zinc ligands as in the LeuP-LAP complex and adjust the hydrophobic side chains of the inhibitors to the S1 binding pocket of the enzyme. Our attempt to perform the simulations of the LeuP-LAP complex without the use of distance constraints led to some distortions of the interactions in the active site of the enzyme, which probably arise from rather poor parameterization of force fields for transition metal ions.

The activities of the generated compounds were estimated using the empirical scoring function (Score_all) included in LUDI [12, 16] and compared with the experimentally observed binding affinities of these inhibitors. Various types of scores, considering different ligand-receptor interactions [17], were calculated:

- Score_all denotes a score value, which is correlated with the predicted inhibitory activity represented by the dissociation constant (K_i) for the ligand-receptor complex, according to the formula:

$$\text{Score\_all} = -100 * \log K_i \quad (1)$$

- Hydrogen_Bond score represents the number and stability of the receptor-ligand hydrogen bonds and is calculated using the geometric characteristic of these interactions.
- Zinc_Score reflects the interactions of ligand with enzyme zinc ion, involving also the geometrical aspects of these interactions.
- Lipo_Score indicates the strength of the hydrophobic interactions between the inhibitor and the enzyme, which depend on the receptor-ligand hydrophobic contact area.

These calculations were performed for two conformations of LAP inhibitors:

- non-optimized structures in the conformation generated by LUDI.
- the structures optimized in the LAP active site, using a CFF97 force field.

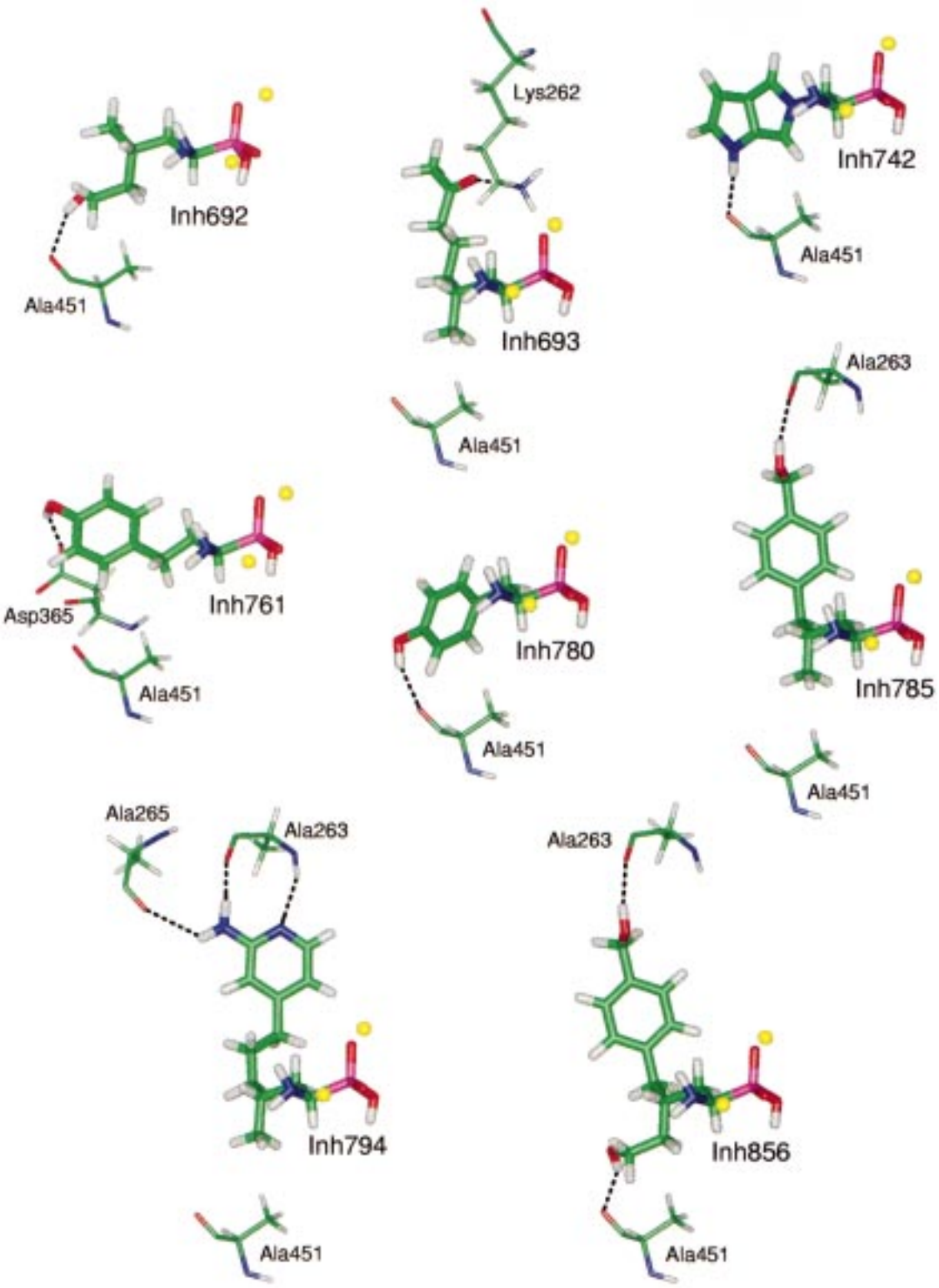


Figure 2. The binding mode of newly designed LAP inhibitors in the S1 binding pocket (the number included in the inhibitor label indicates its Score_all value). The enzyme residues involved in the hydrogen bonds with the inhibitor, as well as Ala451 and the active site zinc ions are shown to facilitate comparison of the inhibitor orientation with respect to the binding of LeuP by LAP, shown in Figure 1.

Similar studies were performed for other known LAP inhibitors, which are LeuP analogues with the modified phosphonic group [7, 19] (the structures of these compounds are presented in Tables 3 and 4). There are three equivalent oxygen atoms in the tetrahedral phosphonic moiety of the LeuP structure. However, the lack of rotation upon binding in the LAP active site makes them enantiotopic (differently located in space and each of them is involved in different interactions with the enzyme) and we described these oxygen atoms as O1, O2 and O3. As a result, taking into account the chirality of the phosphorus atom, appearing upon their binding in the active site of the enzyme, we considered two types of substitution (at the O2 and O3 positions of the phosphonic moiety), replacing these oxygen atoms of LeuP by different substituents (-H, -OCH_3, -CH_3 and -CH_2Cl). The binding mode of these compounds by leucine aminopeptidase is not known, excluding LeuP. For this group of LAP inhibitors, an analysis of the activity was performed for the structures optimized in the active site of leucine aminopeptidase using a CFF97 force field, according to the stepwise procedure described earlier.

Results

The procedures implemented in the LUDI program allowed us to generate numerous potential inhibitors of leucine aminopeptidase. Most of the obtained compounds represent structures for which the activity towards the enzyme has not been measured thus far. However, the most potent aminophosphonic LAP inhibitors were also found (Table 2). Among them the LeuP structure was also reconstructed. The conformation of LeuP generated using the program and its position in the LAP binding site is almost identical with that from the crystal structure of the complex: LAP-LeuP [1]. The superimposition of all heavy atoms of these LeuP structures yielded an RMS value of 0.12 Å.

Among the known LAP inhibitors which were reproduced in our studies, there are mainly aminophosphonic acids obtained by the replacement of the isobutyl moiety of LeuP for other hydrophobic fragments. This is a consequence of the hydrophobic character of the S1 pocket, which binds the side chains of these inhibitors. This pocket is formed by the residues Met270, Ala451, Thr359, Gly362 and Met454 (Figure 1). Therefore, the most effective LAP inhibitors among the analogues of the natural amino acids are the compounds, which contain the hydrophobic side chains. This also may explain why aminophosphonates with short side chains containing polar groups are weak inhibitors of the enzyme [19]. However, based on the search performed using the LUDI program, there are possibilities to find the inhibitors with larger side chains than in LeuP or PheP, because the S1 pocket extends farther, reaching the surface of the protein. Among the designed inhibitors, which can be bound in this pocket, there are both the inhibitors with hydrophobic side chains as well as the compounds containing groups that can be involved in the hydrogen bonds with the enzyme. The binding modes of a few representative structures of these potential LAP inhibitors for which the activity estimated from Equation 1 is higher than for LeuP (Table 1), are shown in Figure 2. The use of LUDI facilitated the finding of the enzyme residues, which may form additional hydrogen bonds with ligands. These residues are placed rather far from the active site of LAP (Figure 2). The inhibitors interacting with them contain a long hydrophobic chain and a terminal polar group. Some of them may form additional hydrogen bonds with water molecules surrounding the enzyme when they reach the surface of the protein. Obviously, they are not analogues of the natural amino acids and based only on the substrate analogy, without the use of LUDI, it would be rather impossible to design these potential LAP inhibitors. The synthesis of these compounds is the subject of our present work and their activity will be determined in the near future.

Table 2 contains different types of LUDI score values for known leucine aminopeptidase inhibitors, which are LeuP analogues differing in the hydrophobic side chains. The largest contribution to their binding energy with LAP originates from the interactions with two zinc ions: Zn488 and Zn489 present in the enzyme active site, which confirms that this type of interaction is the most vital for their binding. Two oxygen atoms (O1 and O2) as well as the nitrogen atom of their aminophosphonate group interact with these zinc ions (Figure 1). Significantly lower are the Hydrogen_Bond score values (about 40–50% of the Zinc_Score values). Three LAP residues are involved in the hydrogen bonds with the aminophosphonate group of the inhibitors: Lys262:HZ1 with the O2 atom, Leu360:O with the O3H group and Asp273:OD1 with the NH_2 group of the inhibitors (Figure 1). The Lipo_Score values for the most active LAP inhibitors studied here ($K_i \leq 1\ \mu M$) constitute about 40–50% of the Zinc_Score values, while these values are significantly lower for the remaining inhibitors of this group.

The values of Hydrogen_Bond and Zinc_Scores for all inhibitors in the conformations generated using LUDI are identical (Table 2), because their aminophosphonic fragments were fixed and thus occupied the same position in the LAP active center. These values are also very similar for the inhibitors optimized in a CFF97 force field with differences up to 10% (Table 2).

The Lipo_Score and Hydrogen_Bond score values for most of the newly designed leucine aminopeptidase inhibitors are higher than for known aminophosphonate inhibitors of the enzyme (Tables 1 and 2), while the Zinc_Score values are very similar for all aminophosphonates studied here.

The Score_all and Lipo_Score values for known LAP inhibitors were correlated with the experimentally determined activities of these compounds ($-\log K_i$) reported in the literature (compounds **1–8** [7], compound **9** [19]) (Figures 3 and 4). The activities were measured under identical experimental conditions, so they are comparable. The inhibition constants for L-LeuP L-PheP enantiomers were used, while the K_i values for the racemic mixtures of all remaining LAP inhibitors were applied, as only such values are available. The score values in each case correspond to L-isomers; however, based on the strong LAP preference towards L-enantiomers of the inhibitors (D-enantiomers of the amino acid analogues are more than 100 times less active than L-isomers), the use of this approximation seems to be applicable, as the observed activity towards the enzyme relates mainly to L forms of the inhibitors.

Quite reasonable results for the correlation of the Score_all values with the experimentally determined activities of the aminophosphonates with different side chains optimized in the LAP active site by a force field approach (correlation coefficient $R = 0.93$) were obtained (Figure 3). The same correlation performed for the structures in the conformations generated by LUDI yielded a lower R ($R = 0.85$) value. The correlation of Lipo_Score with the inhibitory activity for the same group of LAP inhibitors (Figure 4) yielded reasonable results. Similar values of the correlation coefficients for the structures generated using LUDI ($R = 0.93$) and for the structures optimized using CFF97 force field calculations ($R = 0.92$) have been obtained. For these compounds the hydrophobic contacts are essential for their activity differences, which was confirmed by large variation in the Lipo_Score values and reasonable correlation between $-\log K_i$ and Lipo_Score.

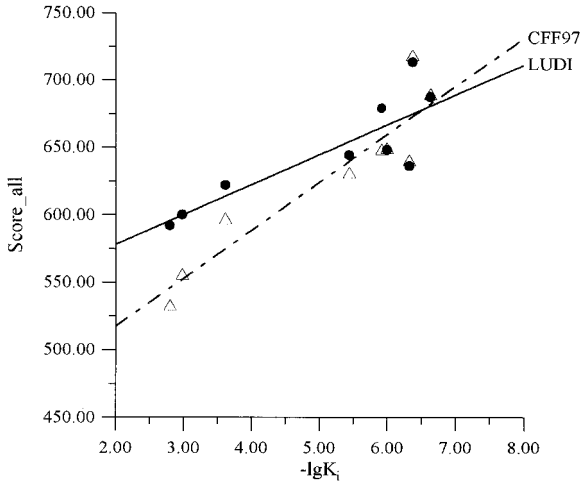


Figure 3. The Score_all values as a function of the activity ($-\log K_i$) for LAP inhibitors: non-optimized structures, generated with LUDI (•, $R = 0.85$) and optimized in the active site of the enzyme, using a CFF97 force field (△, $R = 0.93$).

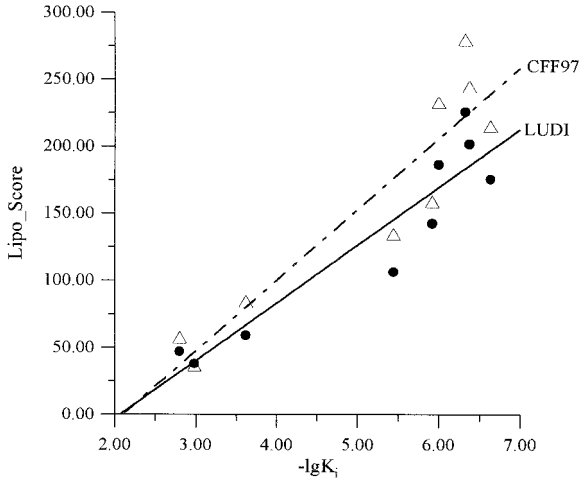


Figure 4. The Lipo_Score values as a function of the activity ($-\log K_i$) for LAP inhibitors: non-optimized (generated with LUDI) structures (•, $R = 0.93$) and optimized by a CFF97 force field (△, $R = 0.92$).

The Score_all values, obtained for the structures optimized using a CFF97 force field, were used here to calculate the binding constants of the inhibitors, according to Equation 1 (Table 2). Almost an ideal agreement between predicted and measured inhibition constants was obtained for LeuP and 1-aminooctanephosphonic acid (compound **3**) and reasonable agreement for the remaining inhibitors, reproduced by LUDI (compounds **2**, **4**, **5**, **6** in Table 2) was also observed. The largest differences between the predicted and measured K_i values have been obtained

for the compounds, which were not found by LUDI (compounds **7**, **8**, **9** in Table 2).

All leucine aminopeptidase inhibitors mentioned above are the analogues of L-amino acids; however, two analogues of D-amino acids (D-PheP and D-SerP) were obtained using the program by the substitution of the hydrogen atom bound to C_α by the appropriate fragments from the LUDI library. Some steric repulsion was observed during the docking of D-PheP and D-SerP in the conformations generated by LUDI into the LAP binding pocket. In order to avoid sterical hindrance, energetically costly distortions of the inhibitor structure, or the enzyme residues are required during the binding to allow their mutual adjustment. This, in turn, reflects in considerably lower activity of D-PheP ($K_i = 15.4$ μM) [7], if comparing with L-PheP ($K_i = 0.42$ μM), and for D-SerP ($K_i = 1580$ μM for DL mixture) [19]. Moreover, it results in very large differences of their activity compared with those predicted by LUDI from Equation 1 (predicted K_i values were 0.05 μM and 4.90 μM for D-PheP and D-SerP, respectively). The finding of only two D-enantiomers remains in the agreement with the substrate specificity of leucine aminopeptidase toward L-enantiomers of the N-terminal amino acids [22], but simultaneously shows that D-isomers which may be localized in an enantiospecifically different mode in the enzyme may also be taken under consideration as potential lead structures.

The calculated score values for the LeuP analogues with the modified phosphonic group and their experimental activities are shown in Tables 3 and 4. For these compounds the differences in their activity do not arise from the hydrophobic, but from the electrostatic interactions with the enzyme (the interactions with the zinc ions and the hydrogen bonds).

The Score_all values for the LeuP analogues with the modified phosphonic group in position O3 change very little, with the differences smaller than 15%, which do not reflect in their activity differences known from experiment (Table 3). The variations of these values are not larger than 15%, compared with LeuP. Moreover, there is no meaningful correlation between the Score_all values and experimentally measured activities of these compounds (for the LeuP-CH_2Cl derivative the K_i value was approximated from the value of the percentage of inhibition).

The Zinc_Score values are very similar to each other in this group of LAP inhibitors, while the Lipo_Score and Hydrogen_Bond Score values do not differ significantly.

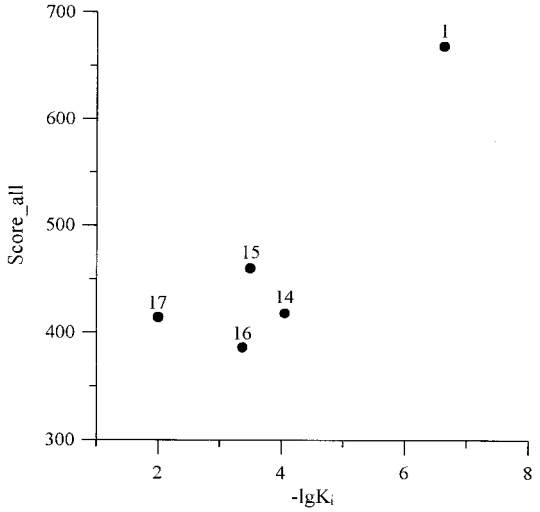


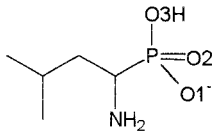
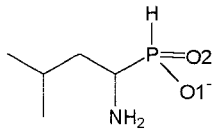
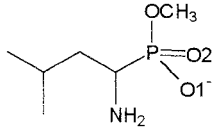
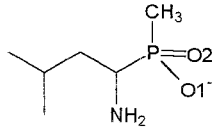
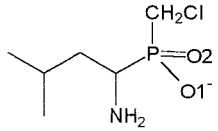
Figure 5. The correlation of the activity with the Score_all values for LeuP analogues with the modified phosphonic group in the position O2 ($R = 0.88$).

The inhibitors with the modification in position O3 of the phosphonic group cannot form the hydrogen bond with Leu360:O, which is observed for LeuP (Figure 1). Because of this, the Hydrogen_Bond score values are slightly lower for them than for LeuP (Table 3).

At the same time, large differences between the Score_all values for the LeuP analogues with the modified phosphonic group in position O2 were obtained. They derive from the absence of the favorable interactions of the substituents placed in position O2 with Zn488 and Lys262 (excluding the LeuP-OCH_3 derivative). The correlation between the Score_all values and measured activities of these compounds can be noticed (Figure 5), but it is not satisfactory ($R = 0.88$).

For this type of substitution, Zn488 loses one of its electron lone pair donors, corresponding to the O2 atom in the LeuP structure. During the optimization of the enzyme-inhibitor system using a CFF97 force field, the decrease of the distance between Zn488 and Asp255:OD1 (previously not involved in the interactions with zinc) was observed. Because of this the Asp255:OD1 atom appears in the coordination sphere of Zn488 (distance < 2.7 Å), while this distance is larger than 3 Å in the crystal structure of LeuP-LAP. The only exception in this situation was found for LeuP-OCH_3 (distance 2.75 Å), which can interact with Zn488 because of the presence of the oxygen atom in the O2 position. This resulted in higher Score_all and Zinc_Score values for this compound, in com-

Table 3. Score values for LeuP analogues with the modified phosphonic group in position O3 together with their activity: experimentally measured and calculated

Inhibitors	Score_all values	Hydrogen Bond Score values	Lipo_Score values	Zinc_Score values	Calculated K_i values (μM)	Measured K_i values (μM)
 **1 (LeuP)**	668	175	213	452	0.208 (L)	0.23 (L)
 **10 (LeuP-H)**	576	107	189	452	1.74 (L)	87 (DL)
 **11 (LeuP-OCH_3)**	583	105	198	452	1.48 (L)	320 (DL)
 **12 (LeuP-CH_3)**	595	140	201	455	1.12 (L)	425 (DL)
 **13 (LeuP-CH_2Cl)**	588	98	234	453	1.32 (L)	20 % of inhibition

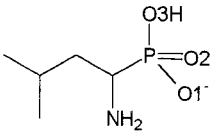
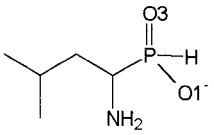
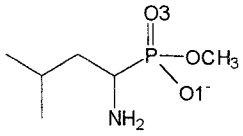
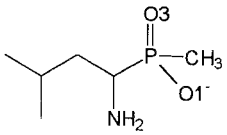
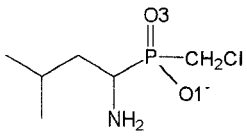
parison with other LeuP analogues. Among the score values for the interactions of these LeuP analogues with LAP, the Zinc_Score values are the highest. The score values for hydrophobic interactions are slightly lower (Table 4) and the lowest are for the hydrogen bonds. This group of inhibitors forms only one hydrogen bond with the receptor Asp273 residue (Figure 1). This contact has a distorted geometry, which results in small values of Hydrogen_Bond Score. This value is also very low for the LeuP-OCH_3 analogue, which has potential possibility for the formation of a hydrogen bond with Lys262, but the geometry of this bond is considerably distorted.

Discussion

The main goal of our work was an application of the Böhm method, implemented in the LUDI program, to design leucine aminopeptidase inhibitors and to predict their affinity towards the enzyme. A very important feature of LUDI seems to be the successful reproduction of the most potent known aminophosphonic LAP inhibitors. These results confirm the usefulness of the Böhm method for the design of enzyme inhibitors.

The values of the inhibition constants calculated using LUDI and measured experimentally are in good

Table 4. Score values for LeuP analogues with the modified phosphonic group in position O2 together with their activity: experimentally measured and calculated

Inhibitors	Score_all values	Hydrogen Bond Score values	Lipo_Score values	Zinc_Score values	Calculated K_i values (μM)	Measured K_i values (μM)
 **1 (LeuP)**	668	175	213	452	0.208 (L)	0.23 (L)
 **14 (LeuP-H)**	418	12	261	317	66 (L)	87 (DL)
 **15 (LeuP-OCH_3)**	460	3	234	398	25 (L)	320 (DL)
 **16 (LeuP-CH_3)**	386	2	240	318	138 (L)	425 (DL)
 **17 (LeuP-CH_2Cl)**	414	6	278	324	72 (L)	20 % of inhibition

agreement for most of the LeuP analogues with different side chains, excluding three compounds (AlaP, GlyP, SerP), which were not generated by the program. Surprisingly, the predicted K_i values for AlaP, GlyP and SerP are significantly lower than those known from the experiment (Table 2). The experimental studies indicate that the L-SerP isomer is significantly weaker bound than the compounds with hydrophobic side chains [7]. This can be attributed to the presence of the polar hydroxyl group in the side chain of this compound, and the lack of the enzyme residues in the hydrophobic S1 pocket, which could be involved in the hydrogen bond with this group. Moreover, the electrostatic repulsion between this inhibitor and the enzyme residues forming the pocket results in low activity of this inhibitor. The differences of the Score_all values between L-SerP and compound **6** (Table 2), which has the methyl group instead of the hydroxyl group, are not as large as could be indicated by their activity differences (K_i value for compound **6** is 439 times lower than for SerP). The empirical scoring function implemented in LUDI was not able to reproduce precisely these activity differences. However, the side chain of SerP could not be generated by the program and placed in the S1 pocket because there are no appropriate enzyme residues to form the hydrogen bond with

the side chain of this inhibitor. This limiting criterion implemented in the program avoids docking of ligand polar groups in the hydrophobic pocket of the receptor. Thus, the LUDI empirical scoring function, used typically for the prioritizing of generated ligands, does not need to consider such effects and seems to be more accurate for the activity prediction of the inhibitors designed and docked by the program. Quite recently, a new version of this empirical scoring function, including additional terms in ligand-receptor interactions, which may improve these effects has been proposed by Böhm [23]. However, this function is not available in the MSI/LUDI_97.0 program.

Moreover, when comparing the experimentally measured activities, we found that AlaP ($K_i = 240\ \mu$M) was significantly less active than compound **6** ($K_i = 3.6\ \mu$M) (Table 2), in spite of small differences in their structures. Thus, the presence of only one additional methyl group in compound **6** increases its activity almost 67 times in comparison to **7**. Such a large difference in the activity, reflecting the presence of an additional methyl or ethyl group, is not observed for other aminophosphonic acids although they are bound in the same binding pocket. This suggests that there is a minimal hydrophobic contact required for proper binding, and further extension of the hydrophobic side chain does not influence the affinity in such a drastic manner. The presence of the considered methyl group in the enzyme binding site could be needed not only to increase the contact area between the inhibitor and the enzyme, but also to 'anchor' the inhibitor in the S1 binding pocket. The side chain of AlaP interacts mainly with residue Met270, while compound **6** is involved additionally in the interactions with Ala451 and perhaps these contacts ensure the 'anchoring' and this could be the reason of such a large activity difference between these inhibitors. Because of the very specific character of such an effect, it is not possible to include it in an empirical scoring function used for theoretical prediction of inhibitor affinity towards the enzyme. These results confirm that the interactions between the inhibitor and the enzyme cannot be fully described if considering them only as additive effects. Moreover, it is worth to emphasize that the energy function implemented in LUDI is designed to reproduce the binding constants, ranging from 2.5×10^{-2} M to 4×10^{-14} M with a standard deviation corresponding to 1.4 orders of magnitude in the binding affinity [13, 15]. The K_i values for three of the weakest inhibitors from Table 2 ($K_i = 240$, 1040, 1580 μM, respectively) lie within this range, but they are at the border of the limit and if we consider the admissible value of the standard deviation, they may be excluded from this range. This may be also an additional reason for the lack of agreement between the predicted and experimental K_i values for these compounds.

Summing up, using LUDI we were able to predict the activity for most of the LAP inhibitors with quite reasonable agreement with the experiment. The present study allowed us to calibrate this approach, because it included the specific effects for this enzyme and may be useful in the estimating of the activity of new LAP inhibitors.

For LAP inhibitors with modified phosphonic groups, the obtained results do not completely explain their binding mode to LAP, although they suggest that the substitution in position O2 of the phosphonic moiety of LeuP will show enantiospecific binding of the phosphonate portion of the inhibitors. Our assumption is based on small variations of the calculated Score_all values for LAP inhibitors with the substitution in position O3, which are insufficiently large, as could be expected from their activity differences as well as from the lack of correlation between the Score_all values and their activity.

Considerably better results were obtained for the LeuP analogues with the substitution at position O2 of the phosphonic group (Table 4). However, there are still some differences between the predicted and measured activities for some of them, particularly for the compounds **15** and **17**. This may arise from the optimization process. The empirical force field may not be well parameterized for some compounds, and it frequently does not reproduce specific details of inhibitor and protein electronic structures [24]. This problem may be even more acute for the transition metal ions in metalloproteins. Thus, the use of this approach may lead in some cases to unrealistic conformations, which then results in incorrect predictions. This limitation may be circumvented in the future by the use of recently proposed novel QM/MM techniques (such as ONIOM, Our N-layered Integrated mO + mM method) [25] or EFP (Effective Fragment Potentials) [26], which may be applied for large molecular systems.

For the inhibitors with the substitution in position O2, the optimization of their structures in vacuo by an ab initio approach was performed and they were docked to the LAP binding site, which remained rigid. However, the correlation of the Score_all values calculated for such conformations of the inhibitors with

their activity ($R = 0.82$) was no better than the results from the force field optimization. This is due to the distorted geometry of the hydrogen bonds and the interactions with zinc ions between the enzyme and separately optimized inhibitors in vacuo, without taking their mutual adjustment into account. This adjustment is particularly important when the analysis of ligand-receptor interactions is based only on the geometrical rules, as it is in the case of the LUDI scoring function, which is very sensitive to changes in distances and angles between the atoms involved in the interactions.

Conclusions

In this paper we describe the computer-aided design of leucine aminopeptidase inhibitors based on the crystal structure of the LeuP-LAP complex. The most active known aminophosphonic LAP inhibitors were reproduced and new potential ones, with higher theoretical activity than those known from the literature, were designed. These new compounds have a shape and charge distribution complementary to the receptor structure and it would be rather impossible to design them without using LUDI or other structure-based approaches. Many aminophosphonates, which were investigated as LAP inhibitors in the past, were designed by analogy with substrate, without knowledge of the receptor structure and none of them was more active than LeuP [7, 19]. Thus, it appears that the new compounds presented in this work may be more potent inhibitors of the enzyme than other known aminophosphonates. Some of them are now synthesised and they will be tested in the future.

The S1 binding pocket of leucine aminopeptidase exhibits hydrophobic character near the active site and the residues which may be involved in the hydrogen bonds with ligands are placed quite far from the active centre. This means that the inhibitors with a long hydrophobic chain and terminal polar group may be bound favourably in this pocket. Moreover, the calculated K_i values for most of the aminophosphonate LAP inhibitors are in reasonable agreement with the experimentally measured ones. Our results confirm that LUDI is a powerful tool for the generation of enzyme inhibitors, predicting their activity and determining the nature of the interactions in the ligand-receptor system. However, some of the systems, particularly with dominant electrostatic interactions, may require a more precise description of the molecular charge distribution and the use of its more accurate representation may improve the inhibitor activity estimates.

Acknowledgements

This work was supported by KBN grants: 6 PO4B 001 15 and 6 PO4A 060 09. The calculations were carried out using hardware and software resources (including the MSI programs) of the Interdisciplinary Center of Mathematical and Computer Modelling at the University of Warsaw (ICM), Supercomputing Center in Poznań, and in the Supercomputing and Networking Center in Wrocław.

References

1. Sträter, N. and Lipscomb, W.N., Biochemistry, 34 (1995) 9200.
2. Taylor, A., Trends Biochem. Sci., 18 (1993) 167.
3. Taylor, A. and FASEB J., 7 (1993) 290.
4. Sträter, N. and Lipscomb, W.N., Biochemistry, 34 (1995) 14792.
5. Pulido-Cejudo, G., Conway, B., Proulx, P., Brown, R. and Izaguirre, C.A., Antiviral Res., 36 (1997) 167.
6. Andersson, L., Isley, T.C. and Wolfenden, R., Biochemistry, 21 (1982) 4177.
7. Giannousis, P.P. and Bartlett, P.A., J. Med. Chem., 30 (1987) 1603.
8. Shenvi, A.B., Biochemistry, 25 (1986) 1286.
9. Sträter, N., Lipscomb, W.N., Klabunde, T. and Krebs, B., Angew. Chem. Int. Ed. Engl., 35 (1996) 2024.
10. Matthews, B.W., Acc. Chem. Res., 21 (1988) 333.
11. Kim, H. and Lipscomb, W.N., Biochemistry, 30 (1991) 8171.
12. Böhm, H.J., J. Comput.-Aided Mol. Design, 6 (1992) 593.
13. Böhm, H.J., J. Mol. Recognition, 6 (1993) 131.
14. Böhm, H.J., J. Comput.-Aided Mol. Design, 8 (1994) 243.
15. Böhm, H.J., J. Comput.-Aided Mol. Design, 8 (1994) 623.
16. Böhm, H.J. and Klebe, G., Angew. Chem. Int. Ed. Engl., 35 (1996) 2588.
17. Ligand Design 97.0 Molecular Modelling Program Package, Molecular Simulations Inc., San Diego, CA, 1997.
18. INSIGHT 97 Molecular Modelling Program Package, Molecular Simulations Inc., San Diego, CA, 1997.
19. Lejczak, B., Kafarski, P. and Zygmunt, J., Biochemistry, 28 (1989) 3549.
20. CFF97 User Guide, Molecular Simulations Inc., San Diego, CA, 1997.
21. DISCOVER Molecular Modelling Program Package, Molecular Simulations Inc., San Diego, CA, 1997.
22. Hafkenscheid, J.C. and Kohler, B.E.M., J. Clin. Chem. Clin. Biochem., 23 (1985) 393.
23. Böhm, H.J., J. Comput.-Aided Mol. Design, 12 (1998) 309.
24. Roterman, I.K., Gibson, K.D. and Scheraga, H.A., J. Biomol. Struct. Dyn., 7 (1989) 391.
25. Gaussian 98, (Revision A.1), Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Zakrzewski, V.G., Montgomery, J.A., Stratmann,

R.E., Burant, J.C., Dapprich, S., Millam, J.M., Daniels, A.D., Kudin, K.N., Strain, M.C., Farkas, O., Tomasi, J., Barone, V., Cossi, M., Cammi, R., Mennucci, B., Pomelli, C., Adamo, C., Clifford, S., Ochterski, J., Petterson, G.A., Ayala, P.Y., Cui, Q., Morokuma, K., Malick, D.K., Rabuck, A.D., Raghavachari, K., Foresman, J.B., Cioslowski, J., Ortiz, J.V., Stefanov, B.B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Gomperts, R., Martin, R.L., Fox, D.J., Keith, T., Al.-Laham, M.A., Peng, C.Y., Nanayakkara, A., Gonzales, C., Challacombe, M., Gill, P.M.W., Johnson, B.G., Chen, W., Wong, M.W., Andres, J.L., Head-Gordon, M., Replogle, E.S. and Pople, J.A., Gaussian, Inc., Pittsburgh, PA, 1998.

26. Day, F.P.N., Jensen, J.H., Gordon, M.S., Webb, S.P., Stevens, W.J., Krauss, M., Garmer, D., Basch, H. and Cohen, D., J. Chem. Phys., 105 (1996) 1968.