Confirmation of Usefulness of a Structure Construction Program Based on Three-Dimensional Receptor Structure for Rational Lead Generation

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Skeletal structures very similar to those of four known inhibitors were automatically output from our computer program LEGEND, based on the three-dimensional structure of the active site of the target enzyme, dihydrofolate reductase. Besides them, the program output novel promising structures that are stable intra- and intermolecularly. This result strongly supports the usefulness of this method for rational lead generation. New lead compounds can be obtained, not relying on chance or trial and error, if appropriate structural selection and modification of the output structures are made.

Introduction

Most lead discovery so far has been performed on the basis of fortuitous finding of biological activities or random screening of natural and synthetic compounds. Stepwise chemical modifications of natural ligand molecules or known drugs can also provide new lead compounds but require extensive repetition of synthesis and evaluation. It is not easy to reach new compounds structurally different from the starting compound.

The rational design to new drug compounds with new skeletal structures needs to be based on the 3D structure of either the receptor or known bioactive compounds. A knowledge of the 3D structure of the receptor protein is far more useful than drug structures, since it can help us to design completely new structures rationally, as well as to interpret structure–activity relationships. Recent progress in various techniques of protein crystallography, protein purification, and gene technology has led to the elucidation of an increasing number of protein structures. Lead generation, nevertheless, remains difficult, because applications have been limited to rather minor structural improvements through docking studies so far, even when the receptor structure is known. So, it seems necessary to establish a general strategy to generate new lead compounds using computers.

There seems to be two main approaches for lead generation using computers. One is searching for structures which satisfy the structural requirements for the activity from structural databases, and the other is constructing new structures so as to satisfy the requirements. In regards to database approaches for the cases where receptor structure is not available, several research groups have developed systems for searching for molecules which match a predicted pharmacophore.[1] They use not only the Cambridge Crystallographic Database but also other 3D structure databases of known and unknown compounds. As a receptor-based database search method, Kuntz *et al.* have developed a unique method focusing on molecular shape, named DOCK.[2] The program DOCK uses a fast sphere-matching algorithm to dock compounds from a user-supplied database in an enzyme active site. DesJarlais *et al.* reported a success with their database approach using the program.[3] They have found that the crystal structure of the antidepressant drug haloperidol can fit well to the substrate-binding site of HIV-1 protease, using the program to screen possible candidates from the Cambridge Crystallographic Database. As a result of assay, inhibitory activities of haloperidol and its analog hydroxyhaloperidol toward HIV-1 and -2 proteases were confirmed experimentally.

The database approaches seem to be very useful for the purpose of lead discovery making the best use of the vast number of accumulated compounds in hand. But, from the viewpoint of covering possible structures as widely as possible, structure construction approaches should be superior to database ones. Furthermore, more favorable intermolecular interactions should be obtainable by structure construction approaches than by searching known compounds. That is, a structure constructed appropriately is like a made-to-order designer dress, whereas the database approach is like choosing a ready-made dress. Several receptor-based methods for automatic structure construction, including our method, have been reported so far.

Lewis and Dean have reported a method for using spacer skeletons, which are assemblies of molecular subgraphs, to obtain molecular graphs that span the binding sites and incorporate predicted ligand points at their vertices.[4] Since then, Lewis has proposed the use of a diamond lattice to determine favorable ways of spanning between distant regions of an active site.[5] Lewis and co-workers have developed a method that treats the atoms in the structures selected by DOCK as an irregular lattice which can be used to connect distant atoms and/or fragments.[6]

Chau and Dean have introduced the idea of combining small (three or four atoms) fragment structures with the chemical graph obtained by Lewis's method.[7-9] Although they have examined various problems such as conformations and charge distributions in structure generation, they have not reported the total system so far.

The LUDI program, reported by Böhm, determines a list of interaction sites into which both hydrogen bonding and hydrophobic fragments are placed.[10] The program uses a library of ~600 linkers to connect up to four different interaction sites into fragments. Then, smaller bridging fragments such as $-CH_2-$ and $-COO-$ are used to link these fragments.

Moon and Howe have developed GROW, which uses a buildup procedure to determine the best peptidal inhibitor or substrate for a given enzyme.[11] A large predefined library of conformations of each amino acid is used in the construction process. Each conformation of each residue is tested according to a molecular mechanics force field.

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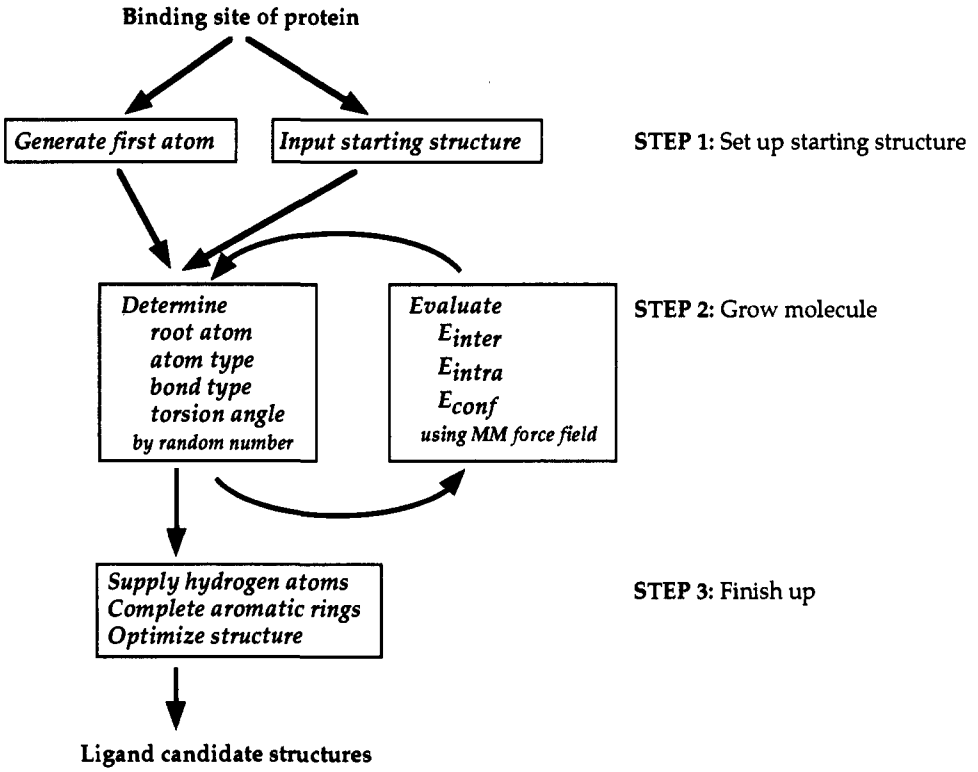

Figure 1. Flow chart of structure generation by LEGEND.

However, the program does not generate general chemical structures but only peptidal ligand structures.

We have developed another method for generating a wide variety of new ligand structures automatically based on 3D receptor structures. The program, named LEGEND, grows molecules by adding atoms one by one up to the specified molecular size using random numbers and force field energy calculation. The program is written in the language C and runs on several UNIX workstations. The algorithm of the program, and a preliminary result to show that the program can generate many possible ligand structures that fit well to the receptor enzyme cavity were reported in 1991.[12]

Since then, we have continued to improve the algorithm and parameters of LEGEND. Two major improvements are made to the algorithm of the previous paper. (1) Heteroatoms such as nitrogen and oxygen are introduced by changing tentatively assigned carbon atoms according to the electrostatic potential value at the new atom position so as to form hydrogen bonds or electrostatic interactions. In the original algorithm, most heteroatoms were not involved in any specific interactions with the receptor because they were introduced randomly. (2) LEGEND has been changed to output only promising structures directly which are selected internally, instead of using another program LORE, because unselected structures have no significance despite the occupation of a vast size of the file space.

Methods

An outline of the structure construction in LEGEND is shown in Figure 1. The algorithm is described briefly in the supplementary material and will be published in detail elsewhere.

As input information, the atomic coordinates of the protein structure are read from a file. The positions of hydrogen atoms and the atomic charge of each atom can be read from the protein file or generated inside LEGEND. The number of molecules to be generated and number of generated atoms in a molecule are also specified at the beginning. First, a rough region on the protein surface which can be occupied by ligand molecules to be newly generated is indicated. Then, inside this region, a three-dimensional grid with a regular interval is generated. At each grid point, van der Waals potential and electrostatic potential are calculated from the whole protein atoms. These potentials are used to estimate the intermolecular interaction energies rapidly and to judge whether a heteroatom is more favorable than a carbon atom at the position or not. The van der Waals potential energy is calculated between protein atoms and a probe atom on the grid point using the MM2 potential function. Several atoms (such as carbon, nitrogen, oxygen, and others) are used as the probe atom, and sets of van der Waals potential energies are stored for all probe atoms separately. Electrostatic potential at each grid point is calculated by using atomic charges of protein atoms.

In regard to the start of structure generation, it is the option of the user either to start from a starting atom which is generated by the program automatically or to start from an input structure. In the former case, the program generates a hydrogen bonding heteroatom so as to form a hydrogen bond to one of the hydrogen bonding groups in the receptor. In the latter case, any fragment structure, such as a partial structure of a known active compound, can be used as a starting structure. Then, atoms are placed one by one, determining a root atom (to which the new atom bonds) and an atom type, a bond type and an atomic position (torsion angle) of the new atom by means of random numbers, using standard bond lengths and angles for corresponding atom pairs. In addition to atom types consisting of a single atom of carbon, nitrogen, oxygen, and others used in the MM2 program, atom types consisting of some fragment structures such as aromatic ring, amide, and carbonyl groups are used in the program. For every new atom, intra- and intermolecular van der Waals energies as well as conformational energies for torsion angle are evaluated using the MM2 force field.[13] If the new atom is not energetically acceptable due to close contact to receptor atoms or previously generated atoms or an unacceptable torsion angle, the program attempts to reassign the root atom. If the attempts fail after a given number of repeats, the program stops trying to add a new atom to the current generated structure and tracks back to the last step; i.e., it withdraws the last of the previously generated atoms and regenerates that atom. For the high-speed estimation of intermolecular van der Waals energy, the tabulated

Table I. Parameters for Structure Generation and Selection

	trial 1	trial 2	trial 3
	Parameters for Generation		
no. of seed atom	4	2	none
hydrogen bond site	$O_{\delta 1}$ (Asp 27)	$O_{\delta 1}$ (Asp 27)	$O_{\delta 1}$ (Asp 27)
atoms per structure	>15	>15	>15
no. of structures	200	200	200
no. of rings	≥2	≥2	≥2
no. of H-bond atom	≥2	≥2	≥2
	Parameters for Selection		
E_{inter}[a] (kcal/mol)	≤−40.0	≤−40.0	≤−30.0
E_{intra}[b] (kcal/mol)	≤100.0	≤100.0	≤100.0
E_{conf}[c] (kcal/mol)	≤20.0	≤20.0	≤20.0

[a] Intermolecular interaction energy (van der Waals and electrostatic). [b] Intramolecular interaction energy (van der Waals and electrostatic). [c] Conformational energy.

data on a 3D-grid are used. Generated carbon atoms on a grid point of very large electrostatic potential are changed into appropriate heteroatoms at a given probability. When the molecule reaches the size specified by the user, the program completes fragmentary aromatic rings by adding missing carbon atoms and supplies hydrogen atoms for all remaining valencies of non-hydrogen atoms. Finally, the structure is optimized by the Simplex method taking into account the intra- and intermolecular energy based on atomic charges calculated by the Del Re method.[14] From the numerous structures generated from LEGEND, a small number of structures are selected on the basis of energetic or other structural criteria.

Preparation

Dihydrofolate reductase is one of the most extensively studied enzymes, and many inhibitor compounds with various structures are known; several are used clinically. Three-dimensional structures of the enzyme from various sources and its complexes with various inhibitors have been elucidated by crystallography. We believed this enzyme system would be a good one with which to demonstrate the usefulness of our program. We chose to use the crystallographic data for the enzyme derived from *E. coli* because the crystal structures of the ternary or binary complex of the enzyme with folate/NADPH, methotrexate, and trimethoprim are known and this is convenient for discussing the results of structure generation.[15-17]

The atomic coordinates of *E. coli* dihydrofolate reductase with NADPH were taken from the crystal structure of the ternary complex of enzyme–coenzyme NADPH–inhibitor folate deposited in the Protein Data Bank.[18,19] Hydrogen atoms were located at the geometrically appropriate positions. Each atom was given an atomic charge value that was taken from that of the corresponding amino acid atom used in the program AMBER.[20] The folate molecule was removed. The NADPH and two water molecules which are greatly stabilized at the bottom of the cavity by more than two hydrogen bonds to the enzyme were left and assumed to be a part of the protein structure. All other water molecules in the PDB file were removed.

The region in which newly generated molecules are to be located was indicated so as to include the substrate-binding site, and a grid of 0.33-Å interval was generated inside the region. Grid point data for van der Waals potential were calculated for carbon, nitrogen, oxygen, and hydrogen. Those for electrostatic potential were calculated with the atomic charges of protein atoms using the dielectric constant, $\epsilon = 1.0$.

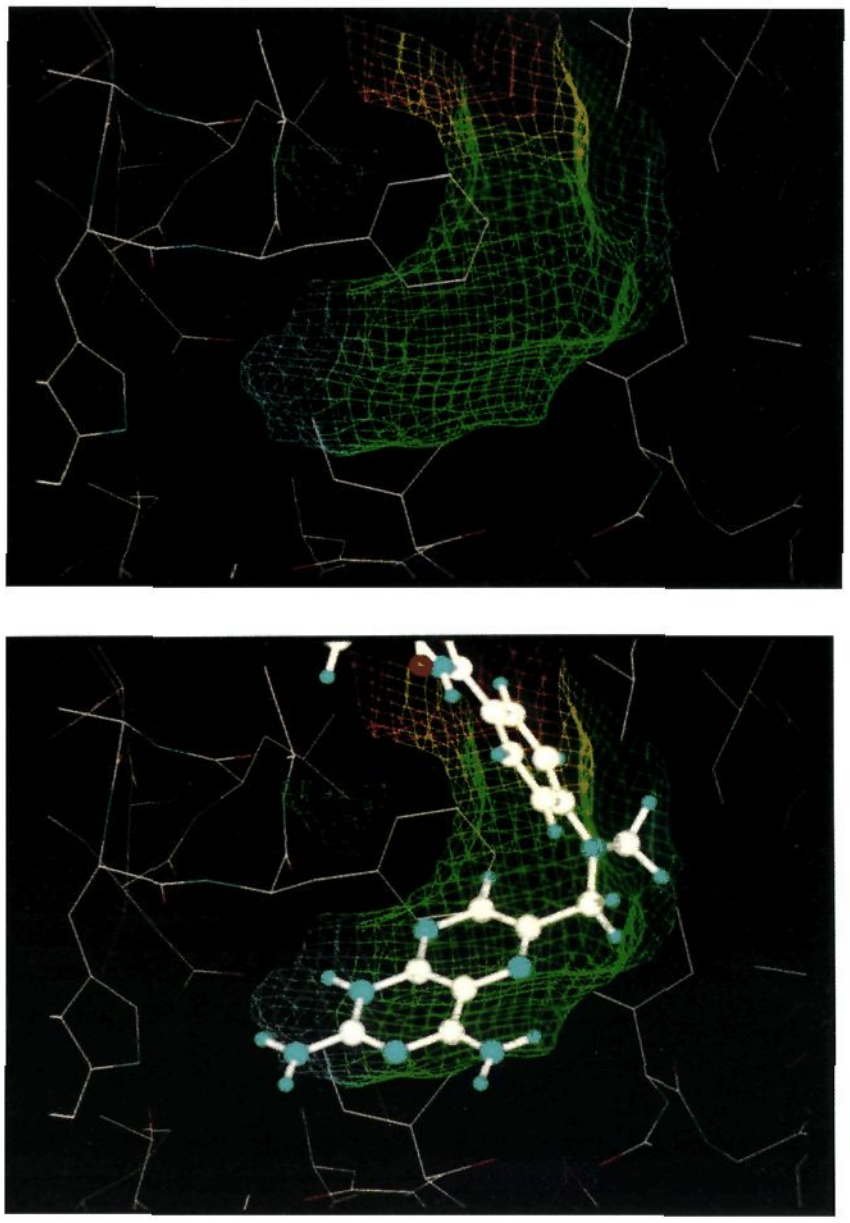

Figure 2. (a, top) The vacant binding site of *E. coli* dihydrofolate reductase. The bird cage is representing ligand atom acceptable region. The color represents electrostatic potential (red: positive, blue: negative). (b, middle) Methotrexate molecule docked in the ligand binding site of *E. coli* dihydrofolate reductase. (c, bottom) A guanidino group used as the starting structure in trial 1.

We carried out three trials of calculation with and without a starting structure. All parameters used in the structure generation and selection are listed in Table I.

Results

The shape and physical environment of the ligand-binding site of the enzyme is shown in Figure 2a. The bird cage expresses the region where a center of a carbon atom is accepted without severe close contact with protein atoms (defined by the GREEN program).[21]

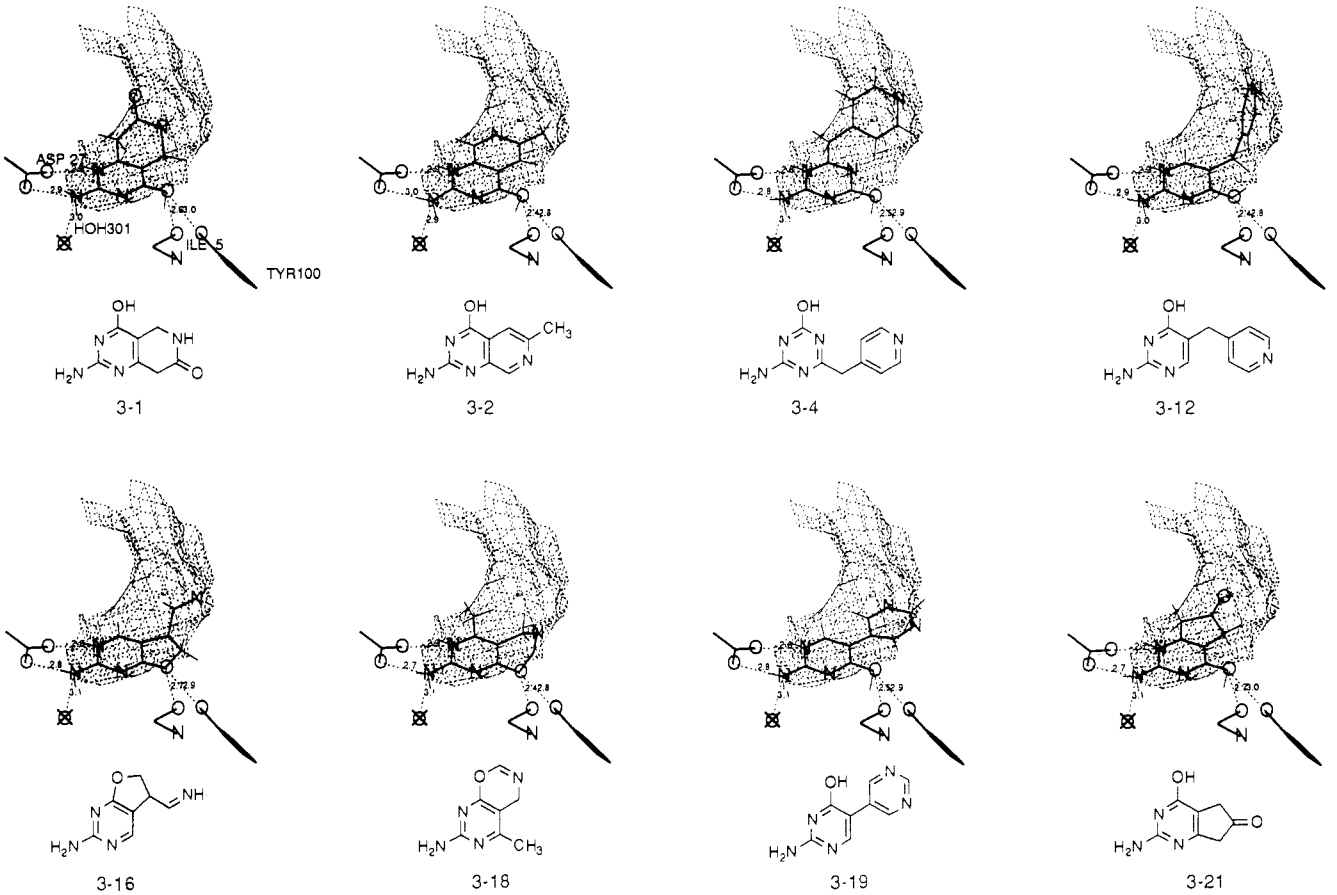

Figure 3. 3D structures and chemical structures of generated structures in trial 1. Structures were optimized in the protonated structures to form stable ionic hydrogen bond with the carboxylate group of ASP 27. Structures with identical skeletons are eliminated.

In the first trial, we aimed to reproduce the skeletal structures of known inhibitors, such as methotrexate, trimethoprim, pyrimethamine, and so on. Most of the known inhibitors for dihydrofolate reductase commonly have a 2,4-diaminopyrimidine moiety in the structure. So, we have attempted to reproduce those structures, using a guanidino group as the starting structure. The two nitrogen atoms and one carbon atom excluding amino nitrogen were assigned aromatic atom types in this trial. This means LEGEND here always generates an aromatic ring including the three atoms, and the orientation of the ring plane is fixed. Because crystallographic data for the enzyme–coenzyme–methotrexate complex are not available in PDB, the group was placed at the same position as that in the methotrexate molecule obtained by docking simulation using our new automatic docking program[22,23] (Figure 2b and c). For simplicity, a rather small molecular size (number of atoms to be generated is 15) was selected. This size seems to be sufficient for the purpose of comparison with the key skeletal structures of the known inhibitors. Only carbon and hydrogen atoms are generated one by one, and carbon atoms on grid points with less than −2.5 kcal/esu or greater than 2.5 kcal/esu were replaced by a heteroatom according to the tabulated probabilities for each heteroatom. Out of 200 output structures, 25 structures (designated as from 3-1 to 3-25) were selected as promising from the viewpoint of conformational and intermolecular stability (20.0 and −40.0 kcal/mol, respectively) and the number of hydrogen bonds (at least 2 hydrogen bonds). The 3D output and the chemical structures of eight compounds with unique skeletons are shown in Figure 3. The 3D structures are shown in a stick representation, together with a bird cage representing the ligand atom acceptable region of the receptor cavity. Plausible hydrogen bonds are shown by dotted lines, and the distances between two heteroatoms are also shown in Figure 3.

It can be easily seen that all these output structures fit well to the receptor cavity and form additional hydrogen bonds besides those of the starting guanidino group. This shows that heteroatoms could be properly introduced to form extra hydrogen bonds by use of random numbers and electrostatic potentials, even though the program does not have a special step to form them.

Among these structures, four key structures of known inhibitors can be found. Although there are minor differences between the output structures and the corresponding known inhibitors such as replacing a heteroatom with a carbon atom or vice versa, output structures 3-2, 3-12, 3-19, and 3-21 are very similar to methotrexate, trimethoprim, pyrimethamine, and TNP-351 (Takeda), respectively, as shown in Figure 4. Figure 5 shows these output structures in the binding site of the protein.

In the second trial, an aromatic amino group, consisting of an amino nitrogen and an aromatic carbon connected to the nitrogen atom, was used as a starting structure. The group was placed at the same position as the 2-amino group in methotrexate used in trial 1. Though an aromatic ring having an amino group is always generated as in trial 1, the ring plane is not fixed in this trial due to a rotational freedom along the nitrogen and the carbon atoms. A total of 25 structures (designated as from 6-1 to 6-25) were selected out of 200 output structures, by the same criteria as in the case of trial 1. Figure 6 shows six independent structures among them.

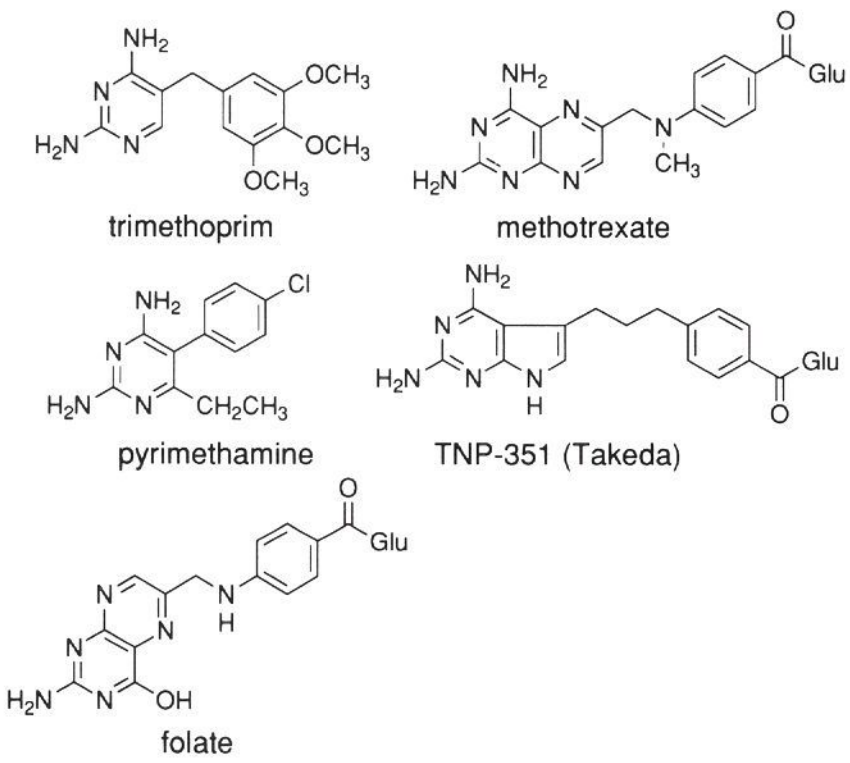

Figure 4. Structures of known inhibitors of DHFR.

In this trial, structures with a guanidino group (2-aminopyrimidine) such as 6-18 or with an amidino group (2-aminopyridine ring) such as 6-4, 6-6, and 6-21 were generated automatically without using them as a starting structure. These groups are known to be easily protonated. But, most of the output structures have an aniline structure, which cannot be protonated by ordinary carboxylic acids. If we accept the hypothesis that protonation to a ligand from the carboxylic acid of Asp27 at the bottom of the cavity is required for the enzymic reaction of dihydrofolate reductase, modifications should be made to these structures from different viewpoints, such as the strength of the corresponding amino groups as a proton acceptor.

In the last trial, we have attempted structure generation without any starting structure. The starting atom was automatically generated so as to form a hydrogen bond to one of the carboxylic oxygens of Asp27. Since only nine structures were chosen from the 200 output structures using the same selection criteria as in trials 1 and 2, rather looser criteria (shown in Table I) were adopted to choose 12 candidate structures (designated as from 7-1 to 7-12). Seven independent structures among them are shown in Figure 7.

In this trial, some structures very similar to the known inhibitors were generated, together with completely novel skeletal structures. In order to obtain a sufficient number of low-energy structures, it is necessary to generate much more structures than 200.

Discussion

A wide variety of stable structures were generated by LEGEND. Key skeletons of most of the known inhibitors were reproduced. This fact strongly indicates that our program can provide valid, promising structures for lead generation. The remaining structures are also expected to be active if they are modified appropriately and synthesized, although we have not yet confirmed the biological activity of any of these candidate structures

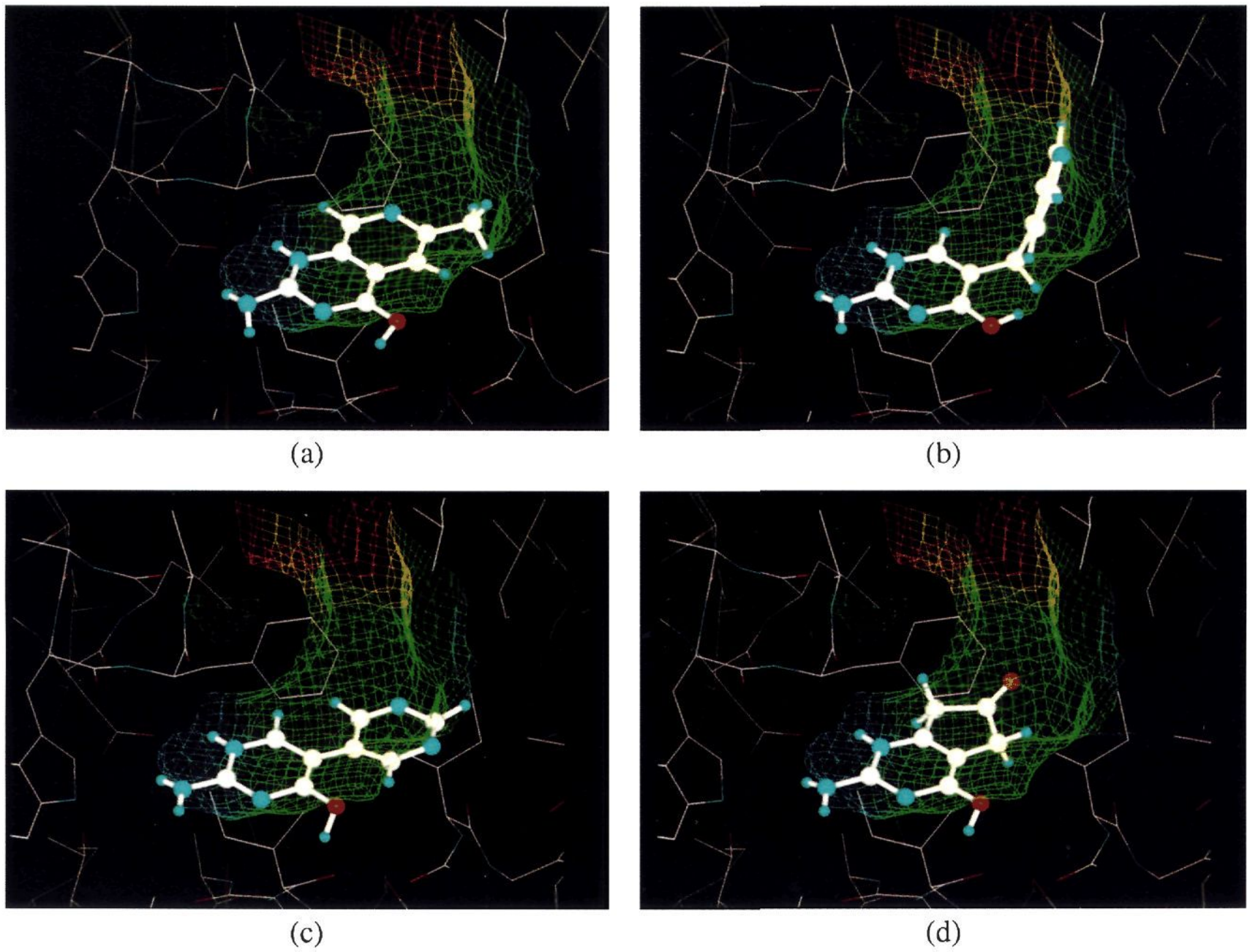

Figure 5. 3D structures of 3-2(a), 3-12(b), 3-19(c), and 3-21(d) in the binding site of DHFR.

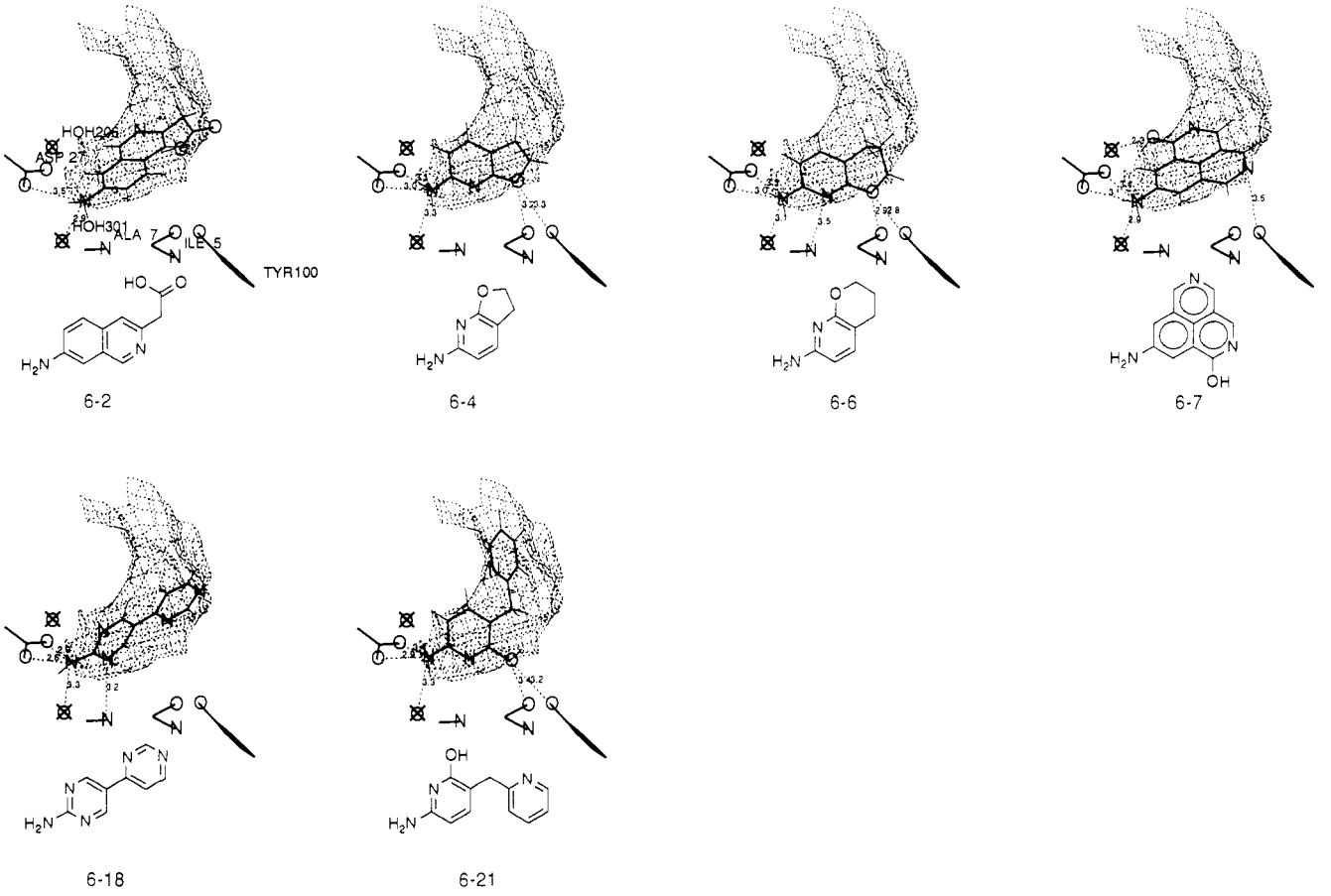

Figure 6. Generated structures in trial 2. Structures with identical skeletons are eliminated.

through chemical syntheses. In order to obtain actual lead compounds, it will be necessary to modify the structures, taking into account physical properties such as solubility, membrane permeability, and so on in addition to improving the receptor binding.

It was naturally found that the smaller the input starting structure, the wider the structural variation of output structures. There may be occasions where hints for new structures are required on the basis of some presumptions, while in other cases, all possible structures should be searched without any presumption. It would certainly be useful for programs to be able to generate all possible structures under a given condition. In addition, it is natural that the larger the selected molecular size, the larger the variation of output structures. But, this kind of diversity is not so important for lead generation. It is the variation of key structures that is important. For example, it is the possible range of structures at the bottom of the receptor cavity that is crucial in the case of dihydrofolate reductase inhibitors. For the purpose of extending these structures to reach the entrance of the cavity, we can design appropriate positions and structures of substituent groups interactively through docking studies, or we can restart from one of the output structures with a promising key structure, using LEGEND.

The validity of the structure construction method should be evaluated from two viewpoints: that is, each output structure itself and its interaction with the receptor protein. The definition of a valid structure is that it must be chemically plausible, geometrically proper, and conformationally not too unstable in the case of a flexible molecule. The former two requirements are easily realized by the introduction of experience-based rules and structure optimization procedures. The present LEGEND uses only a simple rule concerning the atom types of atom pairs which are allowed to bond each other. Although more complicated rules could completely eliminate chemically implausible structures, it will reduce the speed of structure generation. As for the conformation, output structures are regarded as being in the active conformation, in the structure generation methods. So, although the generated conformation need not be the same as that of the energetically global minimum or even that of one of the local minima of a single molecule, it is not desirable that the active conformations are highly unstable compared to the global minimum conformation, since otherwise the activity would not be significantly expressed at low concentration. It might be necessary to examine the relative stability of the output conformation compared with all other possible conformations of the molecule. The active conformation of a drug is not necessarily the same as the conformation found in the crystal or that found in solution. Consequently, in the database search method too, a more favorable conformation (active conformation) should be searched instead of using the crystal structure as it is, although DesJarlais *et al.*[3] did not change the conformations in their successful result described above. Thus, the database search method and the structure generation method have the common problem that the energy difference from the global minimum structure, or the easiness of adopting the conformation, should be considered.

In regard to the interaction of each output structure with the receptor, a good complementary in molecular shape and physical and chemical properties seems to be very important for favorable binding. In particular,

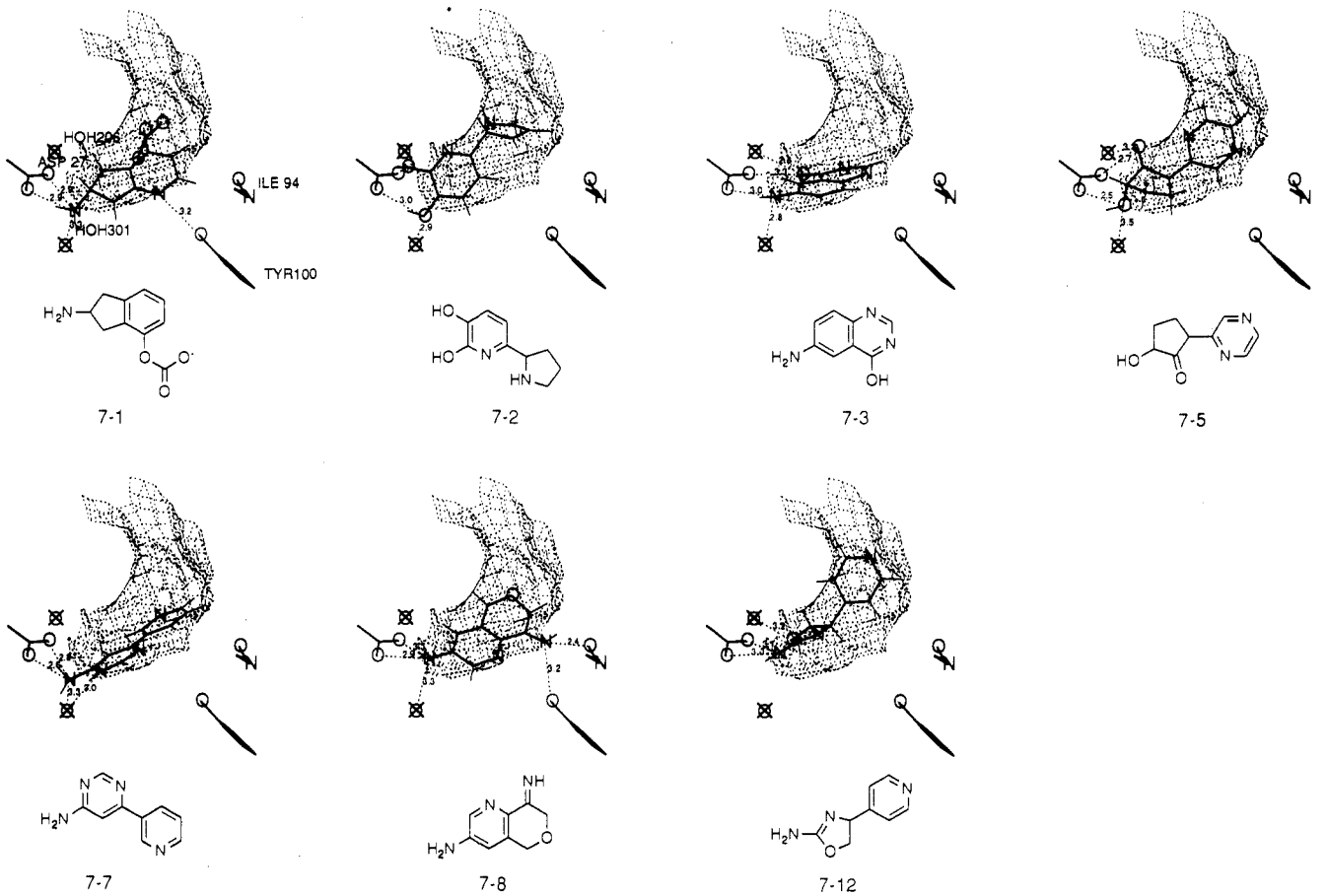

Figure 7. Structures generated in trial 3. Structures with identical skeletons are eliminated.

hydrogen bonds and electrostatic interaction play important roles for strengthening the binding. So, heteroatoms should be generated appropriately so as to match the environment of the receptor cavity as closely as possible in the structure generation process. In the three trials, a considerable number of promising structures were included among the output structures with a consideration of electrostatic potential value of the new atom position to determine the hetero or carbon atom type. Some of the output structures are satisfactorily stable and form hydrogen bonds. But, they occur in low frequency and by chance. It is necessary to take a positive step to generate structures with as many additional hydrogen bonds as possible. We are now improving our program to efficiently output stable structures with more hydrogen bonds and hydrophilic or hydrophobic interactions, reflecting receptor information.

Automatic structure construction can be divided into two classes in terms of fundamental algorithm. One involves growing a molecule atom by atom, and the other involves linking fragments that are prepared in the program.[18-20] LEGEND belongs to the one-by-one category, but partly incorporating the fragment method. The program uses special atom types to introduce moieties such as benzene, amide, and carbonyl groups, as fragment groups. If such an atom type is once assigned as a new atom by random number through the usual atom generating process, the whole group is implicitly introduced into the growing structure with an orientation determined by the next atom position assigned by random number. The basic concept of fragment methods is placing fragment groups at the appropriate positions of the binding site and linking them together. One of the advantages of the simple fragment method is that each generated structure should be chemically acceptable because fragment structures familiar to medicinal chemists are used in the structure generation program. In the case of one-by-one method, the generated structures are not necessarily chemically plausible or acceptable. Therefore, a filtering step to eliminate implausible output structures is necessary in the one-by-one method, and this step reduces the speed of structure generation drastically, as in our program. For example, LEGEND can generate structures in less than a minute per molecule without filtering and structural optimization on the SGI IRIS-4D/35 workstation. However, it requires overnight calculation to generate 200 structures in the actual trials mentioned above. On the other hand, our method is superior to the fragment method in the structural variety of output structures. Each fragment need not be placed at the most stable location, because it is the stability of the whole molecule that is most important. In fragment-based generation, each fragment is placed at the optimum position independently, and this might prevent generation of a stable structure for the whole molecule. The one-by-one method need not place the fragment at the most stable position or orientation. One of the important advantages of the one-by-one method is in the conformation of aliphatic groups. Intermediate torsion angles can be continuously accepted in this method, unless the total stability of the whole molecule is severely decreased. In general, speed of structure generation seems to be incompatible with extensive structural variation.

In one-by-one structure building, there are two possible approaches: random generation and systematic generation. Though only systematic generation methods can

generate all possible structures, the number of them would be too vast to be handled because there are so many degrees of freedom for choosing atom type, atom position, bond type, and so on. We have shown by LEGEND that random generation can efficiently sample various structures and their conformations, although it may not cover all possible structures.

For making use of the generated structures for lead generation, the selection of promising ones for further modification and synthesis is most important. The selection should be done based on both theoretical and synthetic points of view. From the theoretical viewpoint, it is necessary to predict correctly the binding affinity of the output structures to the receptor. But, it is not yet established how to compare or predict the binding stability of various structures with different skeletal structures. Although it is very easy to calculate intra- and intermolecular energy of compounds, the values cannot simply be compared. Empirical energy calculation cannot be used to compare structures composed of different atoms and bonds. Energy values based on molecular orbital calculation have the same problem. Even in very similar molecules in which all other factors can be neglected, the accuracy of force field calculation is insufficient for predicting the relative binding affinity quantitatively. As one order of relative binding constant corresponds to only 1.37 kcal/mol in free energy difference at room temperature, free energy cannot be correctly estimated, even if the enthalpic contribution can be calculated with satisfactory accuracy, because the entropic contribution to the free energy cannot be neglected in many drug–receptor interactions. In order to predict correctly the binding affinity, the free energy difference between the associated state and the unassociated state must be estimated, taking into account the energies for solvation and desolvation. For the time being, it is impossible to order output structures with different skeletons quantitatively. Further progress in fundamental research for free energy calculations is needed.

Supplementary Material Available: Pseudocode description of the algorithm for structure generation used in LEGEND (4 pages). Ordering information is given on any current masthead page.

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