Proposal of a 3D Peptide Pharmacophore of Muramyl Dipeptide-Type Immunostimulants. 1. Conformational Search of Active and Inactive Analogues†

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The CCLUES method [Pristovšek *et al. J. Chem. Inf. Comput. Sci.* **1995**, *35*, 633] is further refined; the set of 42 candidates for the bioactive conformation of the immunostimulator muramyl dipeptide (MDP) obtained in the first run from 1035 originally considered conformations is reduced to the three most probable candidates. The refinement is based on usage of additional information from an inactive and two active analogues: conformations of MDP that superpose well on at least one conformation of the inactive analogue are excluded from the set, while those that do not and additionally superpose well on at least one conformation of the active analogues become more probable candidates. The choice of a final candidate for the bioactive conformation is based on accessibilities of the chemical groups most important for binding to the putative receptor and values of energies calculated with explicit water molecules; the energies of the candidate conformations which differ up to 15 kcal/mol from the lowest-energy conformation *in vacuo* are virtually equalized by the effects of the aqueous medium.

INTRODUCTION

Delineation of the receptor acceptable conformations of a small peptide that may assume thousands of low-energy conformations is a formidable task in the absence of information other than the results of NMR based conformational analysis in solution.[1] However, the identification of what is usually called bioactive conformation is essential for the rational design of peptidomimetics with properties radically superior to those of the natural peptidic lead.

In a previous paper we presented an algorithmic approach (CCLUES[2]) to the problem of reducing the large number of energetically possible peptide conformations to a much smaller number of candidates for the bioactive conformation. The method is based on the assumption that binding to the receptor is mainly accomplished by selected chemical groups of the ligand (the *target atoms*, e.g., hydrogen-bond donors and/or acceptors) and that the bioactive conformation optimally presents the target atoms to the receptor. Inactive analogues possess no conformations that present the target atoms in a similar way because of intrinsic conformational constraints imposed by covalent and/or steric factors. CCLUES searches for a subset of conformations of the active ligand that cannot be attained by any conformation of the inactive ligands in presenting the target atoms to the (putative) binding site of the receptor. A target atom least-RMS superposition value (denoted "RMS superposition") is used for evaluation of similarity of target atom spatial positions;[2] in contrast to Marshall's Active analogue approach[1] it emphasizes information present in inactive analogues and may be referred to as "Inactive analogue approach". The obtained subset of conformations contains candidates for the bioactive conformation and is denoted as the set of structures "unique to the active lead" (in short "unique").

The method is of general applicability; we implemented it to the example of muramyl dipeptide (MurNAc-L-Ala-D-iGln, MDP, **I**, Figure 1; MurNAc: *N*-acetylmuramic acid). The natural lead **I** has been modified for improving its immunostimulant properties; the resulting analogues permitted the establishment of qualitative structure−activity relations[3,4] (SAR). The most important result of SAR concerns the relations of the configurations of the dipeptide moiety with biological activity: the full spectrum of activation of cell defense is deployed only by the L-Ala-D-Glu diastereomer, while the others are either inactive (MDP-L-Ala-L-iGln, **II**, and MDP-D-Ala-L-iGln, **III**) or display activity only under special circumstances (MDP-D-Ala-D-iGln, for instance by incorporation into liposomes though displaying only certain activities[5]). This dichotomy between active (**I**) and inactive diastereomers (**II** and **III**) was most instrumental for the selection of candidates with the CCLUES method; it reduced the initial set of 1035 conformations of **I** to 42 candidates for the bioactive conformation.[2]

However, this is still a number too large for practical purposes of the mimetic design. In this paper we extended the set of analogues subject to the clustering and superposition treatment[2] by one inactive and two active analogues which are used in a refined scheme of the CCLUES algorithm in order to narrow the set of 42 candidates: conformations of **I** that superpose well on at least one conformation of the inactive analogue are excluded from the set, while those that do not and additionally superpose well on at least one conformation of the active analogues become more probable candidates. This resulted in further narrowing of the number of candidates to three. Two of them appear to be less prone to binding on a hypothetical protein binding site owing to their closed structure stabilized by intramolecular hydrogen bonds. The third candidate deploys, in contrast, more potential binding points and is therefore a preferable candidate. However, its in vacuo calculated potential energy exceeds by 5−8 kcal/mol those of the other two conformers. In order to atone this discrepancy we compared the relative energies of the conformers immersed

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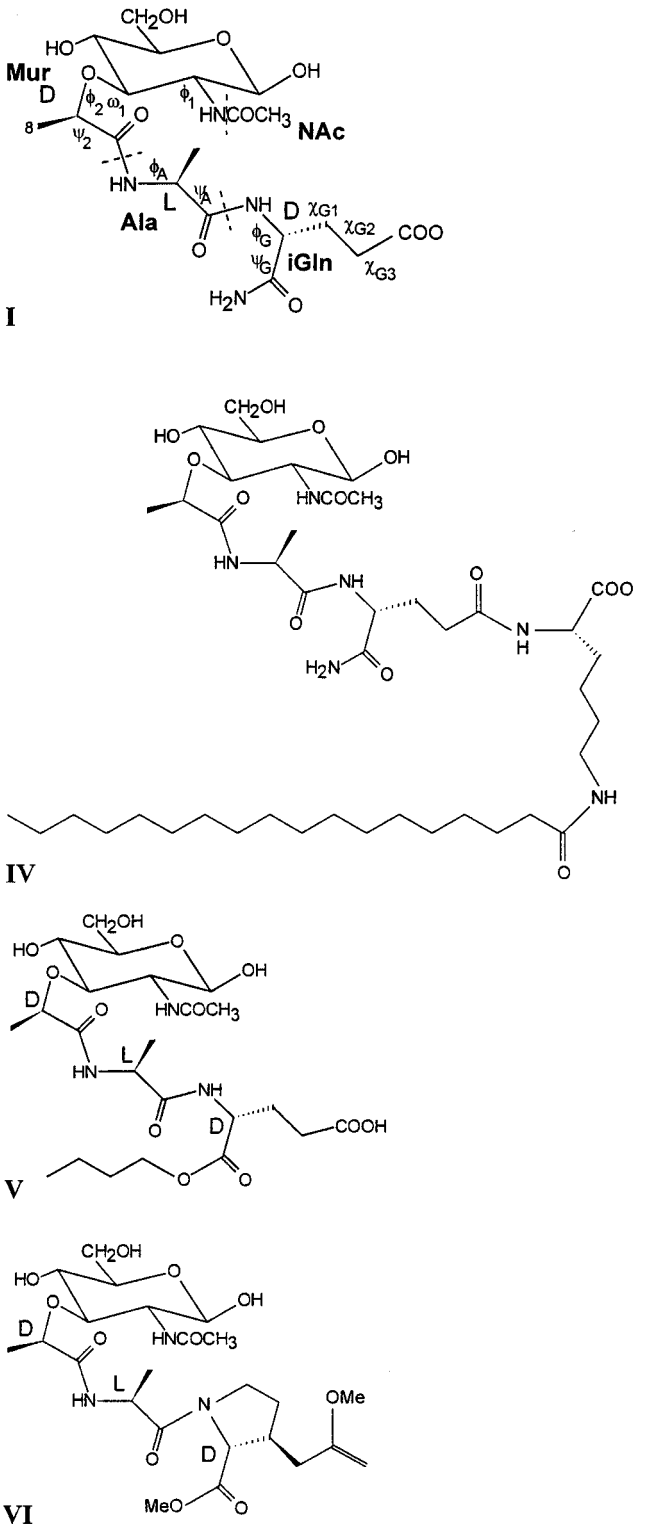

Figure 1. Chemical structures of muramyl dipeptide (**I**), muroctasin (**IV**), murabutide (**V**), and *N*-acetylmuramyl-L-alanyl-3-carbomethoxymethyl-D-proline methyl ester (**VI**).

in water; the former become comparable under this circumstances (come close). A further independent support for the third candidate as the bioactive conformation will be presented in the companion paper; it is drawn from the docking of the peptides to a protein model binding site.

METHODS

The CCLUES procedure[2] is only briefly summarized. An extensive conformational search using the CVFF force field of the program package INSIGHT/DISCOVER[6] was performed for **I**, **II**, and **III** resulting in 4000 energy minimized structures each that were clustered according to the values of the dihedral angles shown in Figure 1. The coordinates of the member with the lowest energy are chosen to represent the cluster; the energy of the cluster is calculated as the Boltzmann averaged total potential energy of the cluster members. The carbonyl groups of the NAc, Mur, Ala, and iGln residues and the side chain carbonyl group of iGln were preselected as target atoms based on SAR data. Each cluster representative j of **I** (j = 1,..,1035, i.e., the number of conformation clusters of **I**, Table 1) was superposed at the target atoms by all cluster representatives of **II** and **III**; the smallest target atom least-RMS superposition value found was stored as jth element of the final vector $\mathbf{V}_{\mathbf{minRMS}}$ (j = 1, ..., 1035). A large value (chosen arbitrarily as >1.20 Å) of $\mathbf{V}_{\mathbf{minRMS}}[j]$ meant that no conformation of any of the two inactive analogues was able to reach the arrangement of the target atoms of cluster j of **I**; the latter was therefore unique to **I** and placed in the set of candidates for the bioactive conformation which finally contained 42 structures (for further details see ref 2).

For this paper the same procedure involving conformational search and clustering is employed for (i) muroctasin (*N*2-[(*N*-acetylmuramoyl)-L-alanyl-D-isoglutaminyl]-N^6-stearoyl-L-lysine, **IV**, Figure 1), a potent analogue with increased lipophilicity,[7] (ii) murabutide[8,9] (**V**, Figure 1), an active analogue with esterified C-terminus, and (iii) the inactive rigidified analogue *N*-acetylmuramyl-L-alanyl-3-(carbomethoxymethyl)-D-proline methyl ester[10] (**VI**, Figure 1) that has already been presented.[2] In the case of **IV** the stearoyl aliphatic chain may influence the sampling of the conformational space *in vacuo* and is replaced by a methyl group.

In the CCLUES procedure the representatives of conformation clusters j of **I** are superimposed at the target atoms with the representatives of the conformation clusters of **IV**, **V**, and **VI**, respectively; the smallest target atom least-RMS superposition values found with each analogue are stored as jth element in the vectors $\mathbf{V}_{\mathbf{IV-I}}$, $\mathbf{V}_{\mathbf{V-I}}$, and $\mathbf{V}_{\mathbf{VI-I}}$, respectively; a large (see below) value of $\mathbf{V}_{\mathbf{X-I}}[j]$ means that no cluster of **X** (**X** = **IV**−**VI**) is able to reach the arrangement of the target atoms of cluster j of **I**. The latter vectors are used for comparison with $\mathbf{V}_{\mathbf{minRMS}}$ using the following two lines of approach: (i) A member of the set of candidates i may be excluded from the set if $\mathbf{V}_{\mathbf{VI-I}}[i]$ is small (see below); in this case the inactive analogue **VI** succeeded in attaining the spatial position of the target atoms of cluster i of **I** which is therefore not unique to the active lead. (ii) On the other hand, a member i advances among the "most probable candidates" in the case that the active analogues **IV** and **V** superpose well the target atoms of cluster i of **I** ($\mathbf{V}_{\mathbf{IV-I}}[i]$ and $\mathbf{V}_{\mathbf{V-I}}[i]$ are small), while **VI** does not ($\mathbf{V}_{\mathbf{VI-I}}[i]$ is large).

The values of the limiting numbers for "large" and "small" RMS superpositions in both cases are arbitrary and are set empirically to 0.8 and 0.5 Å, respectively, compared to 1.2 and 0.3 Å, respectively, in the previous report.[2]

The three final candidates for the bioactive conformation of **I** (cluster 149, 340, and 825) and the representative of cluster 1 presenting the lowest-energy structure found in the CVFF force field are finally selected for thorough examination by a more exact computational method using INSIGHT/DISCOVER.[6]

Table 1. Summary of the Conformational Searches of **I**,[a] **IV**, **V**, and **VI**[a]

	I[a]	**IV**	**V**	**VI**[a]
no. conformation clusters	1035	1753	1702	796
range of energies (kcal/mol)	31.6	57.1	28.1	31.0

[a] Data from the previous report.[2]

Each of the four structures mentioned above is placed in the center of a box with dimensions 15 × 15 × 15 Å and soaked with water molecules. The solute conformation is fixed, while the solvent is relaxed by energy minimization and 6 ps of molecular dynamics at 300 K using periodic boundary conditions (the 6 ps are sufficient to relax the solvent as demonstrated by the nearly constant potential energy in the last two or three ps of the MD runs); the solute is then released and energy minimized in the water bath. The final energies of the solutes and the deviations of the final from the starting structures (calculated as least-RMS superpositions on all atoms) are compared.

RESULTS AND DISCUSSION

CCLUES. A summary of the results of the conformational searches (including the data for **I** and **VI** from the previous paper[2]) is given in Table 1. The smallest number of conformation clusters is found with **VI** since some of its degrees of freedom are restrained by the cyclization in the (former) iGln residue; the largest number and widest range of energies is found with **IV** due to its size. The unexpectedly high number of clusters found with **V** was caused by 77 structures (out of 4000) that had a *cis*-amide bond between Ala and Glu that formed during the conformational search, probably because of the bulky butyl chain at the C-terminus; since they do not affect the CCLUES procedure, no correction was applied.

In Table 2 the values of the vectors $\mathbf{V_{IV-I}}$, $\mathbf{V_{V-I}}$, and $\mathbf{V_{VI-I}}$ are given for the set of candidates for the bioactive conformation of **I** ($\mathbf{V_{minRMS}} \geq 1.2$ Å, see Methods) ordered according to the decreasing values of $\mathbf{V_{minRMS}}$. The narrowing of the set is performed following the two lines of approach outlined in the Methods section.

(i) The column containing the vector elements $\mathbf{V_{VI-I}}$ is checked for low values indicating that the target atoms' spatial position of the representative of the corresponding cluster of **I** can be closely attained by at least one conformation of the inactive analogue **VI**. Values above 1.0 are found most often: nine values are below 0.7 and only one is below 0.5 Å. The value of the RMS superposition that is small enough to allow exclusion from the set of candidates could not be set exactly. In the previous paper[2] the lower limit of $\mathbf{V_{minRMS}}[i] \leq 0.3$ Å was used for inclusion of **I** cluster *i* in the set of "common clusters" which denoted spatial positions of target atoms common to active and inactive analogues; cluster *i* was then classified as being excluded from consideration as a candidate for the bioactive conformation. In the case of **VI** the same limiting value was not used since **VI** is not only a diastereomer of **I** but also possesses additional chemical groups that pose additional requirements for space in the binding site. The lower limiting value was therefore increased to 0.5 Å; two clusters of **I** could be excluded from the set of candidates, namely no. 856 ($\mathbf{V_{VI-I}}[856] = 0.51$ rounded down) and 520 ($\mathbf{V_{VI-I}}[520] = 0.48$); both are marked in the last column of Table 2. The question arises why the procedure in the previous report[2] involving the two inactive diastereomers has not excluded the two conformation clusters from the set of candidates in the first place. A possible answer would be insufficient conformational search of **I**−**III** that is the first step of the CCLUES procedure; another explanation would take into account the conformational restraints of **VI** that enforce certain positions of the target atoms not encountered with **II** and **III**.

(ii) In the second approach, the columns 4−6 of Table 2 are scanned for large values of $\mathbf{V_{VI-I}}$ and small values of $\mathbf{V_{IV-I}}$ and $\mathbf{V_{V-I}}$; the conformations of **I** in such a row are the most probable candidates. Once more the limiting values of the respective vector elements had to be chosen; the lower limiting value denoting similarity of the target atoms' spatial position of the two active analogues was set to 0.5 Å as in the previous case, while the upper limiting value denoting dissimilarity for the inactive analogue was lowered from 1.20 to 0.8 Å due to similar reasons. With these values three of the candidates were extracted and focused on as the most probable candidates, namely clusters no. 149, 340, and 825; they are outlined in Table 2 including a comment describing intramolecular H-bonding (or its absence) in the structure which is given for comparison with the conformational analyses in solution.[8,11] The stereoviews of the structures are presented in Figure 2.

The first (no. 149) is a compact structure with the lowest energy of the three (7.7 kcal/mol); in the Mur-Ala moiety the pseudo-β turn characterized by the hydrogen bond *N*-Acetyl-CO-HN-Ala is found. The pseudo-β turn is a specific conformational feature in solution and is again encountered among the most probable candidates for the bioactive conformation. In the previous paper[2] it was found also among the common clusters and is well accessible to inactive diastereomers.

The other two structures are more extended and compute to higher (relative) energies of 10 and 15 kcal/mol, respectively, in the CVFF[6] force field. The second of the three candidates forms a γ-turn in the Ala residue which is not observed in solution,[8] while the third has no intramolecular H-bonding and has the lowest values of $\mathbf{V_{IV-I}}$ and $\mathbf{V_{V-I}}$ (0.27 and 0.36 Å, respectively).

All three outlined structures have nearly the same values of the dihedral angles ϕ_1 (from −103 to −109°), ψ_1 (from 65 to 67°; being part of the sugar ring it is necessarily constant), and ω_1 (from −99 to −107°), while the angles ϕ_2 and ψ_3 are nearly the same for the extended structures 340 and 825 ($\phi_2 \approx 135°$, $\psi_3 \approx 30°$) and distinctly different for the pseudo-β turn containing structure 149 ($\phi_2 = 157°$, $\psi_3 = -76°$). The dihedral angles ϕ_1 and ω_1 in the muramyl moiety are therefore well determined by the CCLUES procedure and may be fixed in further search for a 3D pharmacophore; most valuable is the information regarding the angle ϕ_1 since the latter completely describes the position of the *N*-acetyl group relative to the sugar ring.

Accessibilities of Target Groups. Energies. The first of the three most probable candidates for the bioactive conformation of **I** yielded by the CCLUES procedure is a structure containing the pseudo-β turn which is the most distinguished conformational feature in solution according

Table 2. Clusters with the Largest Values of $\mathbf{V_{minRMS}}$ and the Corresponding Values of $\mathbf{V_{IV-I}}$, $\mathbf{V_{V-I}}$, and $\mathbf{V_{VI-I}}$ (See Text)[d]

cluster no. of **I**[a]	energy[b] (kcal/mol)	$\mathbf{V_{minRMS}}$ (Å)	$\mathbf{V_{IV-I}}$ (Å)	$\mathbf{V_{V-I}}$ (Å)	$\mathbf{V_{VI-I}}$ (Å)	comment[c]
80	6.1	1.51	0.87	0.72	1.49	
296	9.6	1.37	0.77	0.77	1.37	
7	2.6	1.37	0.85	0.82	1.30	
14	3.3	1.37	0.76	0.20	1.48	
218	8.7	1.37	0.72	0.58	0.82	
182	8.2	1.36	0.74	0.72	1.44	
278	9.4	1.35	0.79	0.17	0.69	
96	6.6	1.34	0.70	0.12	1.47	
243	9.0	1.34	0.76	0.70	0.89	
809	14.9	1.33	0.47	0.79	1.39	
756	14.3	1.32	0.64	0.68	0.62	
598	12.9	1.31	0.63	0.64	1.39	
109	6.9	1.31	0.70	0.53	1.35	
642	13.2	1.30	0.51	0.81	0.62	
401	10.9	1.30	0.96	0.89	1.02	
116	7.0	1.29	0.53	0.66	1.34	
90	6.4	1.29	0.77	0.69	1.24	
403	10.9	1.29	0.71	0.76	1.18	
816	14.9	1.29	0.32	0.42	0.64	
202	8.5	1.28	0.64	0.48	0.66	
436	11.2	1.28	0.34	0.61	1.24	
609	12.9	1.27	0.51	0.90	1.42	
506	11.9	1.27	0.84	0.81	1.02	
149	**7.7**	**1.27**	**0.39**	**0.47**	**1.14**	**NAc-CO-HN-Ala**
856	15.6	1.27	0.55	0.59	0.51	excluded!
176	8.1	1.26	0.94	0.61	1.37	
340	**10.1**	**1.26**	**0.30**	**0.49**	**0.81**	**Mur-C9O-HN-iGln**
744	14.1	1.26	0.75	0.61	0.93	
688	13.6	1.25	0.64	0.74	1.13	
637	13.2	1.24	0.72	0.53	1.04	
226	8.8	1.23	0.87	0.56	0.71	
212	8.6	1.22	1.12	0.43	1.39	
257	9.1	1.22	0.86	0.71	1.28	
770	14.5	1.22	0.67	0.75	0.64	
629	13.1	1.21	0.82	0.76	1.55	
413	11.0	1.21	0.50	0.67	1.32	
474	11.6	1.21	0.60	0.24	0.60	
520	12.0	1.20	0.53	0.81	0.48	excluded!
927	17.1	1.20	0.87	0.71	1.01	
361	10.3	1.20	0.72	0.60	0.77	
825	**15.1**	**1.20**	**0.26**	**0.36**	**0.91**	**no. intram H-bond**
203	8.5	1.20	0.46	0.59	1.22	

[a] Serial number of conformation cluster sorted by increasing energy. [b] Boltzmann average of the potential energies of the cluster members (relative to the lowest energy found that is set to 0.0). [c] Contains information for the most probable candidates regarding intramolecular H-bonding; structures excluded from the set of candidates are marked with "excluded!". [d] In bold: the lines containing the three most probable candidates for the bioactive conformation if **I**.

to NMR data. Globally, the structure is contracted ("folded", see Figure 2, top) which may be clearly attributed to the *in vacuo* conditions during simulations.[12] In this structure one of the target atom groups which is of key importance (NAc-CO) is not exposed to the putative receptor because of its involvement in intramolecular H-bonding; the same, to a lesser extent, is true for Ala-CO in cluster no. 340 which is involved in a γ-turn. In the third, most extended candidate structure (Figure 2, bottom) all target atoms are accessible. This makes the first two of the three candidates the less probable ones which is in contrast with the energy criterion that favors them.

Because of this discrepancy and, moreover, the relatively high values of the CVFF energies of the final candidates of **I** (7−15 kcal/mol) the energies of the latter have been recalculated in a water box using explicit solvent molecules and periodic boundary conditions; the same calculation has been performed with the representative of cluster 1 (relative energy of 0.0 kcal/mol *in vacuo)* that presents the lowest-energy structure found during the conformational search with the CVFF force field (Table 3). For comparison the lowest of the four resulting energies has been set to 0.0 kcal/mol; because of the included intermolecular interactions with the solvent molecules the absolute values of the energies should not be compared with the former set calculated in *vacuo*. The relative energies of the minimized structures in the water bath range from 0 to 3 kcal/mol and are therefore nearly equalized in comparison with the much larger differences *in vacuo*; the representative from cluster no. 1 remains lowest in energy but is closely (0.5 kcal/mol) followed by cluster no. 825 (computed with 15 kcal/mol *in vacuo*). These results show that a conformational search of polar ligands should be preferably conducted with explicit polar solvent; unfortunately, such computations are not feasible in reasonable time. As a consequence, the possibility of the existence of conformations possessing much lower energy in explicit water cannot be excluded; however, the main point questioning the relevance of energy values in the search for the bioactive conformation remains valid.

The changes in the conformations during energy minimization in the water box are insignificant (0.1−0.3 Å RMS

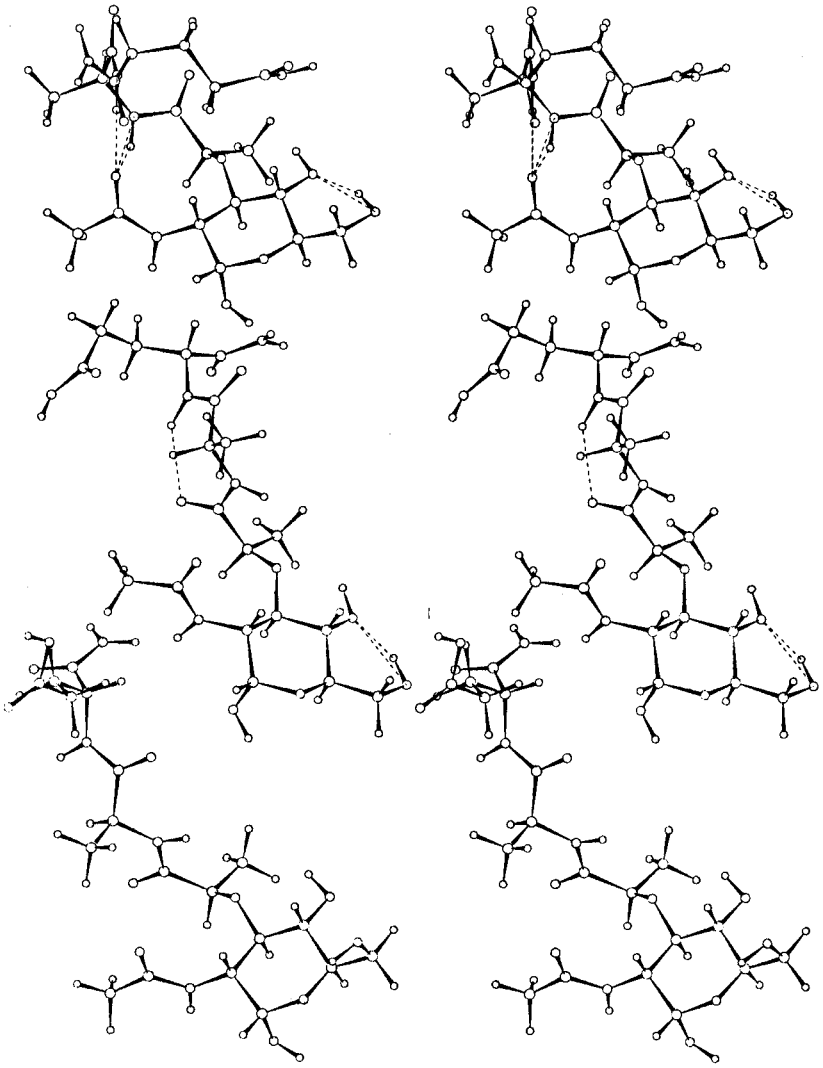

Figure 2. Stereoviews of the representatives of conformation cluster no. 149 (top), 340 (middle), and 825 (bottom) of **I**.

Table 3. Potential Energies (in kcal/mol) of the Representatives of Selected Conformation Clusters of **I** in Vacuo ($\epsilon = r$) and Using Explicit Solvent (Water) Molecules[e]

cluster no.[a]	E_{CVFF}[b]	E_{CVFF} in water box[c] (RMS[d])
1	0.0	0.0 (0.11)
149	7.7	2.9 (0.33)
340	10.1	1.5 (0.20)
825	15.1	0.5 (0.20)

[a] Serial number of conformation cluster sorted by increasing energy. [b] DISCOVER CVFF[6] potential energy, copied from Table 2. [c] In a 15 × 15 × 15 Å water box (868−870 solvent molecules); force field as in *b*. [d] The RMS deviation (in Å) of the final from the starting structure. [e] The values are given relative to the lowest one (that is set to 0.0 kcal/mol) found in each case.

on all atoms) and allow the minimized structure to remain in the same conformation cluster they started from. The latter point is important from the point of view of the CCLUES method; if the latter structures had left the cluster they originated from, their relevance as candidates would have to be assessed anew.

These results show that large differences in energies calculated *in vacuo* do not and should not exclude the higher-energy structures obtained during conformational searches from consideration as either possible structures in solution or, even more important, as possible candidates for the bioactive conformation; searching among low-energy structures *in vacuo* for bioactive conformations of flexible molecules may be futile and should be extended to higher relative energies. This finding also justifies the little weight given to the CVFF energy values of candidates in the decision process of the CCLUES procedure; although immensely useful because of their speed of calculation, the energies returned by the empirical force fields, especially without inclusion of a solvent medium, should be interpreted and used with caution. These conclusions are entirely in agreement with the results of a systematic study by Nicklaus *et al.*[13] which reports that the bound conformation of a flexible ligand is not usually any of the low energy minima found *in vacuo* and that the protein-bound conformation may be found virtually anywhere on the energy surface. It is therefore imperative for the protocol used for conformational search to ensure a fair exploration of conformational space even in the higher energy region.

With these cautions in mind we decide upon the representative of cluster no. 825 (Figure 2, bottom) as the final most probable candidate for the bioactive conformation; the choice is based on the accessibility of its target atoms and the comparable value of its potential energy in the water box. Neither of the two other candidates is dismissed from consideration but is given less weight.

CONCLUSION

The CCLUES method is rather qualitative and user-dependent since it employs limiting values of RMS superpositions that are assigned arbitrary values. Additionally, it is based on certain suppositions and simplifications; it depends on thorough sampling of the conformational spaces of each molecule and the correct choice of target atoms; it applies purely geometric criterions for activation of the putative receptor binding site and neglects the possibility of close contacts of some atoms of the superposed analogues with the latter.[2] Therefore the set of candidates derived by the method has to be treated with caution. The same is true for narrowing of the set and the final most probable candidates; they should possess reasonable potential energy and expose the target atoms to the putative receptor. All these conditions are met by the final candidate from cluster no. 825. Due to the limitations of the method listed above and, particularly, to the choice of critical contacts with the putative binding site, the results should be checked by an independent method before attempting to build the first pharmacophore of MDP-type immunostimulants. The most reasonable checking of the contacts can be carried out by investigating the binding to proteins that specifically bind MDP; such an independent method is presented in the accompanying paper that uses the T4 Iysozyme peptide binding site as a model for the binding site of the putative MDP receptor.

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