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Generation and Evaluation of Putative Neuroregenerative Drugs. Part 2: Screening Virtual Libraries of Novel Polyketides which Possess the Binding Domain of Rapamycin

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Abstract—The use of computational methods to direct engineered biosynthesis toward candidates based on the desired properties of the target compounds has been explored. The objective for this study has been the modification of rapamycin in order to eliminate its immunosuppressive activity and retain its neuroregenerative abilities. We have designed analogues of rapamycin which have truncated effector domains but retain the ability to bind to FKBP proteins, which is a prerequisite for the neuroregenerative abilities of the drugs. The procedures described here consist of the screening of large virtual libraries of molecules which retain the binding domain of rapamycin but in which different substitute ketide units replace the effector domain. These methods have provided analogues of rapamycin that cannot retain the immunosuppressive abilities of rapamycin, have a binding affinity to FKBP12 identical to that of rapamycin (by linear interaction energy calculations), and are suitable for synthesis by modified polyketide synthases.

Introduction

Polyketides are natural products whose biosynthesis takes place through successive Claisen condensations of simple carboxylates, e.g. acetate, propionate, or butyrate. Naturally occurring polyketides include a large number of bioactive molecules including antibiotic, antifungal, and antitumor drugs. Polyketides can vary widely in size, from the eight carbon 6-methylsalicilic acid up to maitotoxin which, with a molecular weight of 3422, is one of the largest natural products known.[1] Polyketides can contain aromatic rings, branched chains, polycycles, and a variety of other structural features.

The biosynthesis of polyketides shares many characteristics with the biosynthesis of fatty acids. The ketide chain grows by two carbon atom increments during each condensation cycle, and modifications to each ketide unit follow immediately after the condensation step. Unlike fatty acid synthesis, the reduction of the β-carbonyl group in the newly added ketide unit is not required following the condensation step. As a result, the polyketide chain can contain a combination of carbonyl groups, alcohols, double bonds, or methylene units. Additionally, successive condensations can add a variety of different carboxylates, resulting in a large number of possible substituents with varied stereochemistry. This variability in the nature and modification to the substituents results in a virtually endless number of possible polyketides.

The enzymes responsible for the biosynthesis of polyketides are called polyketide synthases. The polyketide synthases of interest in this study are modular enzymes, in which multiple catalytic units (modules) are assembled into a cluster responsible for the total synthesis of the polyketide chain.[2] Each module catalyzes a specific reaction, forming a molecular assembly line in which successive modifications contribute to the formation of the final product. The cloning of the polyketide synthase responsible for the synthesis of erythromycin provided the first insight into the nature of a modular polyketide synthase (PKS), as well as the first opportunity to modify their function.[3,4] Modifications performed on the erythromycin PKS include modifications to the oxidation states of the ketides in the macrocycle, deletions of ketide units, and the inclusion of synthetic non-ketide units through the interruption of the polyketide synthesis, as has been summarized in reviews by Katz[2] and

Abbreviations: PKS, polyketide synthase; SASA, solvent accessible surface area; LIE, linear interaction energy.

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Khosla.[5] Future studies on PKSs hold the promise of breaking new ground in the engineering of biomolecules.

Ligands that bind to the protein FKBP12 have attracted attention in recent years due to the neuroregenerative abilities described for several such ligands.[6] The best known FKBP12 ligands are the polyketides FK506 and rapamycin, which bind in an identical way to FKBP12 through structurally similar regions of the drugs known as the binding domain (Chart 1).[7] Most synthetic FKBP12 binders contain the pipecolinic and dicarbonyl portions of the binding domains of rapamycin (Chart 2) and FK506. A comparison between the structures of several unnatural FKBP12–ligand complexes (pdb entries 1fkg, 1fkh, and 1fki)[8] and the complexes of FKBP12–FK506 (pdb entry 1fkf)[9] and FKBP12–rapamycin (pdb entry 1fkb)[10] was performed in this laboratory. We found that the conserved parts of the binding domain bind in an identical fashion in the complexes of FKBP12 with the unnatural ligands as with the polyketides.

Rapamycin is synthesized by a modular polyketide synthase (PKS). The biosynthetic gene cluster for rapamycin has been cloned.[11] Three clustered genes were found to be responsible for generating the rapamycin PKS, which together encode 14 modules of the PKS cluster. Each unit in the cluster was found to be responsible for a single round of chain elongation, and an adjacent gene was found to encode a pipecolate incorporating enzyme which completes the macrocycle. In all, 70 constituent active sites are involved in the biosynthesis of rapamycin. Chart 3 contains a schematic drawing of the ketide units added by each module of the PKS cluster. The thick bonds indicate atoms that are added to the product in each module, starting with the cyclohexyl ring in the cyclohexyl arm (Chart 1) and progressing toward the pipecolate ring (Chart 3, from work by Schwecke et al.[11]).

The putative neuroregenerative abilities of rapamycin analogues capable of binding to FKBP12 without forming ternary complexes with FRAP have raised considerable interest. In a previous study,[12] we examined the effect that deleting ketide units from the effector domain of rapamycin had on the binding affinity of the resultant ligand to FKBP12 and on the formation of the ternary FKBP12-rapamycin–FRAP complex. That study showed a marked drop in the binding affinities of modified rapamycin ligands to FKBP12 when the ligand effector domain became smaller than five ketide units in length. The drop became more noticeable as the ligand became small enough to bring the substituents on the effector domain close to the binding domain. This raised the question of whether it was feasible to create novel analogues of rapamycin or FK506 in which the effector domain of the native drugs is completely replaced. The

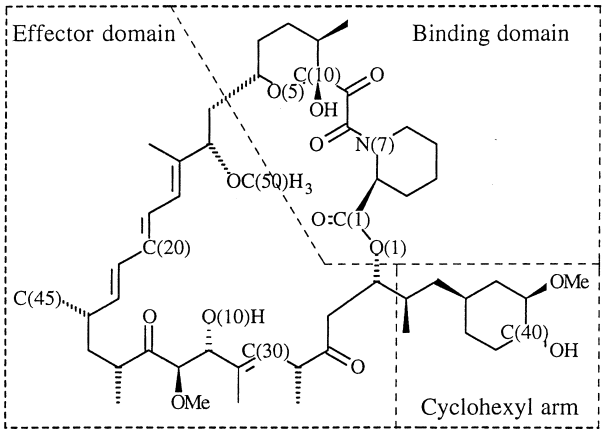


Chart 1. The structure of rapamycin divided into binding domain, effector domain and cyclohexyl arm as described in the text. The atomic numbers shown correspond to the standard numbering of atoms in rapamycin.

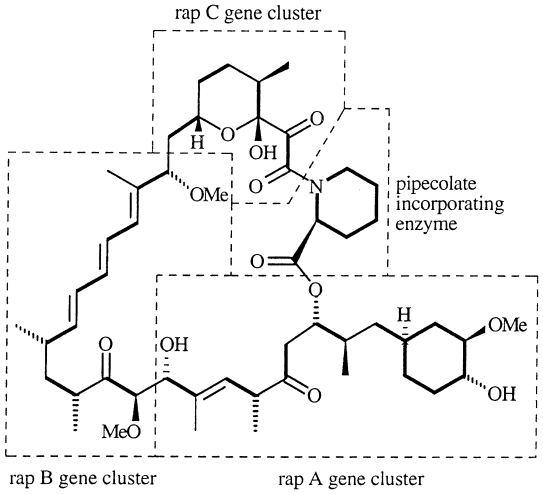


Chart 2. Rapamycin divided up according to the biosynthetic gene clusters responsible for generating the drug. The thick bonds in the structure correspond to the individual ketide units added at each step by the polyketide synthases. The biosynthesis starts at the cyclohexyl ring in the rap A gene cluster and progresses toward the pipecolate ring.

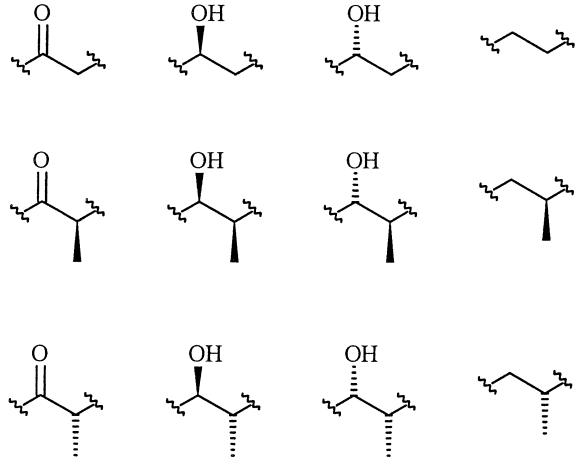


Chart 3. The cassette of ketide units considered in the combinatorial substitutions. The methylene ketide unit (top right) was used as a template in generating the initial conformations, and subsequent substitutions corresponded to replacing the methylene units with the other units in the chart.

replacement effector domain would be a short polyketide tether with substituents placed in such a way to favor holding the binding domain of the ligand in a conformation ideally suited for binding to FKBP12. The benefits of these smaller tethered rapamycin analogues, over direct modifications to the native drug, could be several. The novel ligands would be too small to protrude from the surface of FKBP12, thereby avoiding the possibility of binding to an effector protein. This method could also yield novel FKBP12 binders with very high affinities, since the binding domain of the ligand would be properly prepositioned to bind to FKBP12.

In this study, we examine the arrangements of ketide units which favorably form an effector domain (tether) that can replace the natural effector domain of rapamycin and have a significantly reduced size relative to the parent drug. We have carried out the following: (i) Remove the ketide units encoded by the 'rap B' frame in the biosynthetic gene cluster for rapamycin, thereby creating a rapamycin analogue with a shortened effector domain (tether); (ii) perform zero, one, or two deletions from the 'rap A' frame in the gene cluster (Chart 2), which results in the three different tether lengths examined in this study; and (iii) finally replace each of the ketides in the tether with each of the 12 ketides in Chart 3 in a combinatorial fashion.

The selection criteria applied in finding putative neuroregenerative agents are: (a) The binding domain of the ligands must favor an orientation that is complimentary to the FKBP12 binding pocket. (b) The tether linking the two ends of the binding domain must not favor a position in which it protrudes into areas occupied by FKBP12. (c) The modified ligand must favor a conformation of the cyclohexyl arm (Chart 1) corresponding to that of rapamycin bound to FKBP12. Unlike our earlier study [12] of modified rapamycin ligands, no consideration has to be given to the formation of ternary complexes with the effector proteins FRAP or calcineurin, since the sizes and shapes of the novel ketides bear no resemblance to the effector domains of the parent drugs.

Methods

The following approach was applied in building and evaluating the virtual libraries employed here. A more detailed explanation of each step is provided later in this section. (1) Create analogues of rapamycin in which the effector domains were shortened and the cyclohexyl arm (Chart 2) was removed. (2) Create template ligands in which the modified effector domains of the rapamycin analogues **I**, **II** and **III** were replaced by methylene chains. (3) Perform a stochastic conformational search on the templates**,** yielding a conformational library for each of them. (4) Perform combinatorial substitutions on each of the conformations in the conformational libraries, yielding libraries of polyketides. (5) Perform potential energy calculations on each of the polyketides. (6) Identify conformations that are suitable for binding to FKBP12 and determine the energy of that conformation relative to other conformations of that polyketide. (7) Evaluate the free energy of binding for the best ligands using molecular dynamics (MD) interaction energies scaled using the linear interaction energy (LIE) approximation.

(1) The effector domain of rapamycin was altered by removing the rap B' part in Chart 2, yielding the novel rapamycin analogue **I**. Additionally, one and two of the ketides from the 'rap A' part were deleted, giving rise to the rapamycin analogues **II** and **III**, respectively. The cyclohexyl arm of rapamycin (Chart 2) was replaced with a methyl group. The rapamycin analogues **I**, **II** and **III** are shown in Chart 4.

(2) The modified effector domains of **I**, **II** and **III** were replaced by methylene chains of equal length, giving rise to the templates **IV**, **V** and **VI**, which are shown in Chart 5.

(3) Stochastic conformational searches[13,14] were performed on the templates **IV**, **V** and **VI**. Starting structures were generated in two distinct conformations of the C–N amide bond in the binding domain. The starting structures were subjected to 5000 Monte Carlo pushes using the program MM3(94).[15] The stochastic searches yielded pools of 1200–3000 conformations from each template. Conformations differing by at least 0.5 Å in RMS fit between all non-hydrogen atoms for template **IV** and by at least 0.3 Å for templates **V** and **VI** were retained in the pools. The conformation pools contained 349 conformations for template **IV**, 2223 conformations for template **V**, and 1,416 conformations for template **VI**.

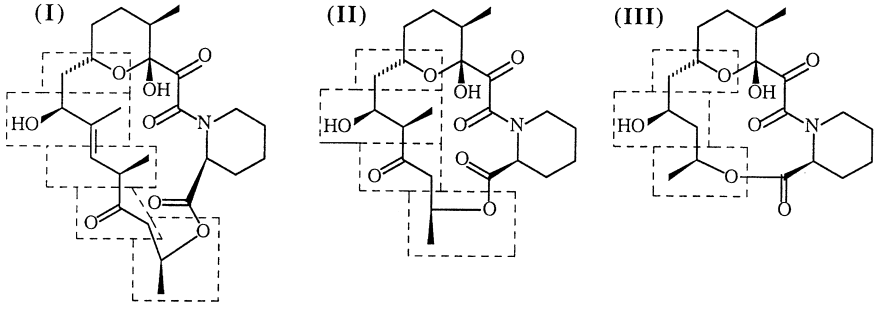
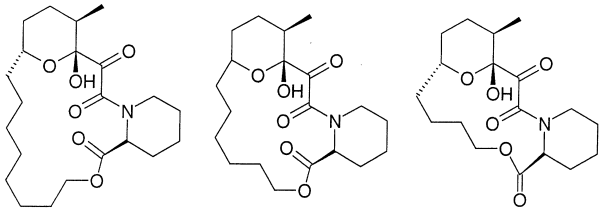


Chart 4. Examples of the substituted rapamycin analogues examined in this study. The dotted lines indicate the division of the tethers into individual ketide units, showing where substitutions were performed to generate novel tethers.



(IV) (V) (VI)

Chart 5. The template ligands generated by replacing the effector domains of the ligands in Chart 4 with methylene chains.

(4) Combinatorial substitutions were performed on each of the conformations to be considered using the cassette of ketide units in Chart 3. This yielded libraries of 3.6 million pentaketides derived from template **IV** (349 conformations and 10,836 substitutions), 1.9 million tetraketides derived from template **V** (2223 conformations and 864 substitutions), and 101,952 triketides derived from template **VI** (1416 conformations and 72 substitutions). As an example, the 72 substitution patterns derived from template **VI** are shown in Chart 6.

(5) Potential energies were calculated for each of the polyketides generated in part (4) using MM3(94). For each substitution pattern, the energy values were used to calculate the probability of formation for each conformation relative to other conformations of the same polyketide using Boltzmann's equation for population distributions (eq (1)):

$$P_i = \frac{e^{-\beta E_i}}{\sum_j^N e^{-\beta E_j}} \quad (1)$$

where P_i is the probability of finding the molecule in conformation i out of the N conformations considered, and β is $1/(kT)$, where k is the Boltzmann constant and T is the temperature.

(6) Suitable FKBP12 binding conformations for each polyketide were identified by comparing the conformation of the binding domain to the binding domain of rapamycin when bound to FKBP12. Each polyketide that favored conformations in which the binding domain differed by less than 0.5 Å from the rapamycin binding conformation was subsequently docked in the binding site of FKBP12. Conformations that did not fit into the binding site were discarded.

(7) Representative polyketides with favorable conformations were evaluated using the interaction energies from molecular dynamics (MD) simulations scaled using the linear interaction energy approximation. MD simulations were carried out using Amber 5.0.[16] All MD simulations were performed in a periodic water box that extended at least 9 Å from every atom in the protein–ligand complexes. A constant dielectric ($\varepsilon = 1$) was used, the non-bonded cutoff was set to 8 Å, 1—4 van der Waals interactions were divided by 2.0, and 1–4 electrostatic interactions were divided by 1.2. Separate temperature scaling was used for the solvent and solute, the time constant for coupling to the external heat bath was 0.01 ps, the temperature was set to 300 K, the time step was set to 1 fs, and the non-bonded pairlist was recalculated every 25 steps. During equilibration runs, bonds involving hydrogens were constrained using the SHAKE algorithm[17] and bond interactions involving hydrogens were omitted. During production dynamics runs, all interactions were calculated and the SHAKE algorithm was, therefore, not used. The ligands were assigned Gasteiger–Marsili charges.[18] The OC–CO bond of the α-ketoamide was assigned new parameters giving it no torsional preference (the rotational preference is due to steric and electrostatic interactions). This reproduces the HF/6-31G(d) torsion energy profile for *N,N*-dimethyl-3-hydroxyl-2-oxopropionamide, from Gaussian 98.[19] The energy profile has a maximum at 0° O–C–C–O bond rotation, a local energy maximum at 180°, and two energy minima at ±110° bond rotations (Fig. 1). The location of the conformational minimum calculated using these parameters differs by roughly 25° from the ab initio energy profile.

Each ligand–FKBP12 complex was surrounded by a periodic water box that extended at least 9 Å from all atoms in the protein–ligand complex, resulting in box sizes of roughly 63×63×57 Å. The system was energy minimized for 200 steps using steepest descents and 9800 steps using conjugate gradient optimization. The density of the surrounding water box was equilibrated during a 10 ps constant pressure MD run holding the protein and ligand atoms fixed in space. An additional 10 ps MD equilibration simulation was performed at constant volume without geometric constraints but using SHAKE for bonds involving hydrogens. Finally, constant volume molecular dynamics were run for 100 ps on the equilibrated binary FKBP12–ligand complexes. The effects of the ketide deletions on the relative binding energies of the ligands to FKBP12 were evaluated from the average van der Waals interaction energy between the ligand and FKBP12. The interaction energies were estimated relative to the energies of the free ligands in a water box, which were run in an identical manner as described for the ligand–protein complexes.

Solvent accessible surface areas (SASA) for the ligand–FKBP12 complexes were calculated based on the positions of non-hydrogen atoms using the program Naccess.[20] The probe radius used was 1.4 Å and the slice thickness was

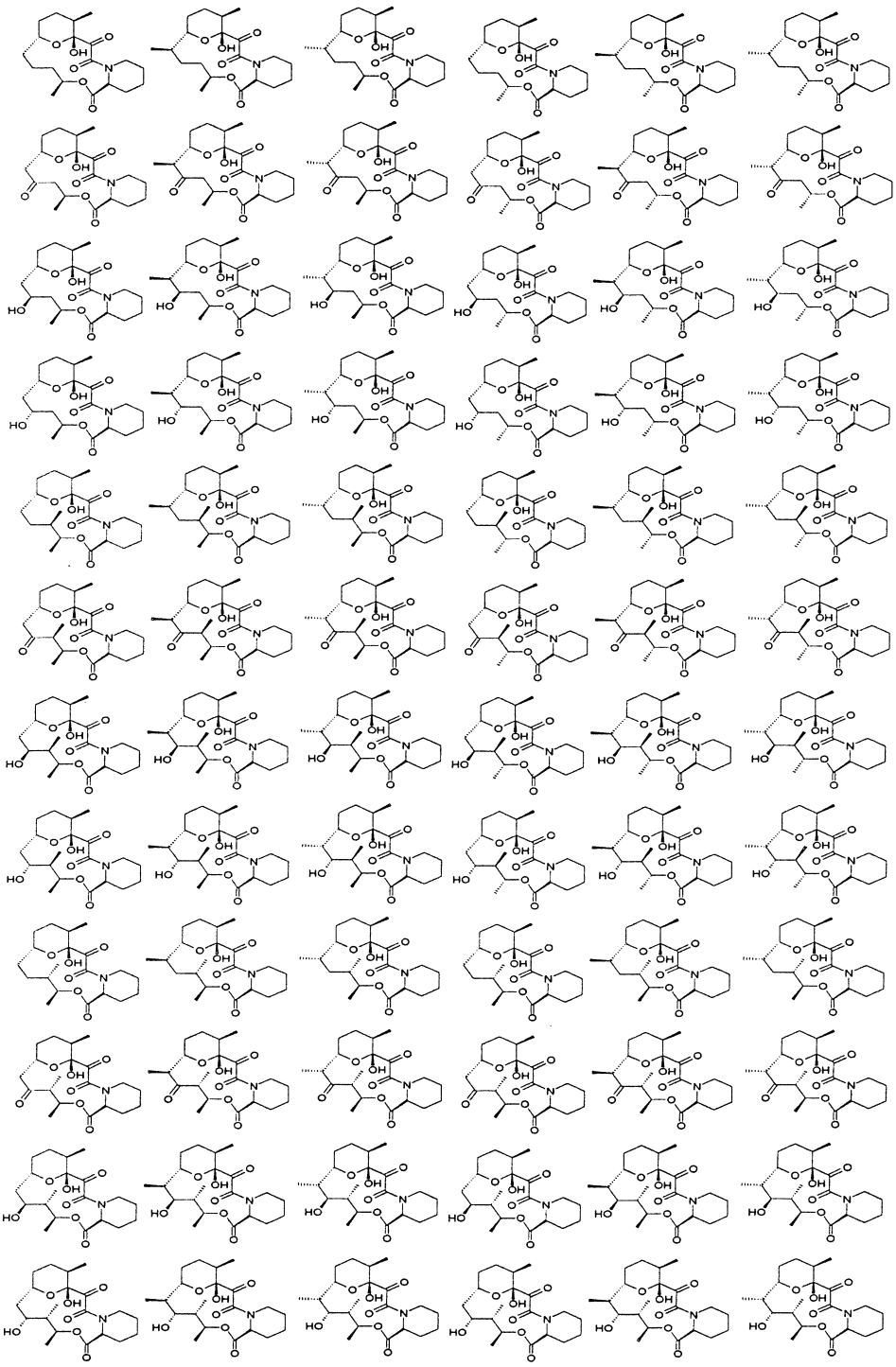


Chart 6. The substitutions performed on template **VI**.

0.05 Å. The SASA of the ligands and the surrounding amino acid residues was used as a measure of whether the MD simulation had reached equilibrium at the start of production dynamics. All residues that had at least one atom within 5 Å of rapamycin in the binary rapamycin–FKBP12 complex were used in the SASA calculation. These were the amino acid residues Tyr26, Gly28, Phe36, Asp37, Phe46, Gln53, Glu54, Val55, Ile56, Arg57, Trp59, Tyr82, His87, Ile90, Ile91, Leu97, and Phe99. The flexibilities of the protein backbone atoms in the binary complexes were determined from the least squares fit deviation of the backbone from the average structure during 100 ps MD runs. These can be compared to the crystallographic Debye–Weller B-factors (assuming that the B-factors are free from contributions from lattice effects and that the motions of atoms are isotropic) using the relationship $\langle \Delta r^2 \rangle^{1/2} = (3B/8\pi^2)^{1/2}$.[21] Gaps formed between the ligand and

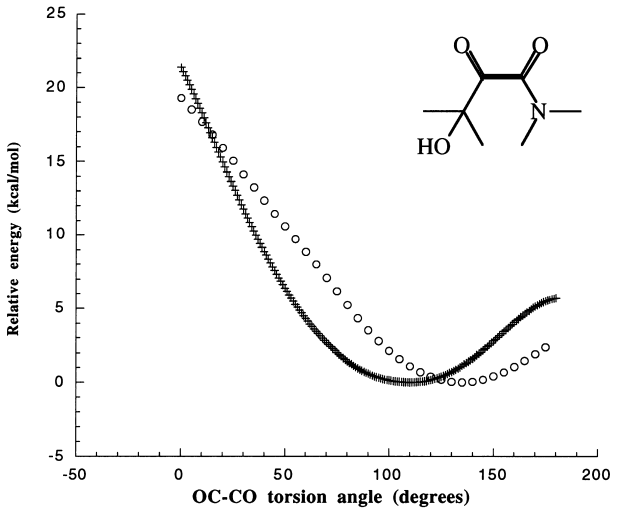


Figure 1. A comparison of the torsional energy barriers for bond rotation around the C–C(=O)–C(=O)–N bond in *N,N*-dimethyl-3-hydroxyl-2-oxopropionamide calculated using HF/6-31G(d) (o) and the Amber parameters devised for this study using PM3 charges for the 2-oxopropionamide (+).

protein during the simulation were calculated using the program Surfnet.[22] The gap calculations utilized a 6×6×6 Å boundary around the atom range, a grid separation of 0.8 Å, an initial sphere size of 1 Å, a maximum sphere size of 4 Å, and a scaling factor of 1.0.

Åqvist's 'linear interaction energies' (LIE) method[23,24] was used to calculate the free energies of binding of rapamycin and the modified ligands to FKBP12. Åqvist's method is based on the assumption that, using molecular dynamics simulations, the free energy of ligand binding to a protein target can be expressed using the equation

$$\Delta G_{\text{bind}} = \alpha_{\text{prot}}\langle V^{vdW}_{\text{l-s}}\rangle_{\text{bound}} - \alpha_{\text{wat}}\langle V^{vdW}_{\text{l-s}}\rangle_{\text{free}} + \beta_{\text{prot}}\langle V^{el}_{\text{l-s}}\rangle_{\text{bound}} - \beta_{\text{wat}}\langle V^{el}_{\text{l-s}}\rangle_{\text{free}} + \gamma \quad (2)$$

where α_{prot} and α_{wat} are scaling factors for the average potential van der Waals energies $(< V^{vdW} >)$, β_{prot} and β_{wat} are scaling factors for the average potential electrostatic energies $(< V^{el} >)$, γ is an empirical constant to reproduce experimental free energies of binding, and the subscripts prot and wat refer to the FKBP12–ligand complex in water and the free ligand in water, respectively.

In this study, we use the parameters that gave good general fits in Åqvist's study,[24] which are $\alpha_{\text{prot}} = \alpha_{\text{wat}} = 0.163$, $\beta_{\text{prot}} = 0.348$, $\beta_{\text{wat}} = 0.340$ and $\gamma = -1.89$. Snapshots were taken at 1 ps intervals from the MD studies of the FKBP12–ligand complexes and free ligand simulations. Potential energies (van der Waals and electrostatic) were calculated for the ligand at each snapshot conformation, and the average potential energies were used in calculating the free energies of binding from the LIE equation.

Results and Discussion

Triketides

The template **VI** contains the shortest modified effector domain that can link the two ends of the binding domain while still retaining a conformation similar to that of rapamycin bound to FKBP12. Our earlier study[12] indicated that the binding affinities of ligands of this size to FKBP12 are weak.

The stochastic conformational search on **VI** revealed that the binding domain cannot assume the orientation found in rapamycin. The lowest energy conformation that closely resembles the required binding orientation has a least squares fit of 0.36 Å for the picolinyl and tetrahydrofuranyl rings. Comparison of **III** and rapamycin showed that the two molecules exhibit a close fit (0.06 Å) between the picolinyl rings of the two ligands, but the tetrahydrofuranyl ring of **III** is rotated 35° relative to rapamycin. Acceptable binding conformations found for **III** (less than 0.5 Å least squares fit to the rapamycin binding conformation) have a combined probability of 1.7%.

The rapamycin analogue **III** was docked in the binding domain of FKBP12 and an MD run was performed on the complex in water. The average gap between **III** and FKBP12 is 207 ± 44 Å^3, which indicates that **III** covers a very small portion of the protein surface. This is also evidenced by the SASA of the residues near the binding pocket, which is 727 ± 83 Å^2, comparable the 989 Å^2 SASA of these residues in the absence of **III**. The LIE binding energy of **III** to FKBP12 is −3.5 kcal/mol, 3.1 kcal/mol less favorable than was found for rapamycin.

For the 72 novel polyketides based on template **VI** (Chart 6), conformational searches reveal a wide spread in conformational preferences depending on tether substituents. Bar plots for two representative energy distributions are shown in Figures 2 and 3 (the binding conformations with the lowest energy are indicated by arrows in the figures). The bar plot in Figure 2 is for the triketide **VI-A**, which strongly favors having the α-keto amide of the ligand effector domain in the opposite orientation from the binding conformation of rapamycin (Fig. 4(a)). The bar plot in Figure 3 is for triketide **VI-B**, which single point energy calculations predict should have an 80% probability of existing in conformations that had a least squares fit to the binding conformation of rapamycin of less than 0.5 Å (Fig. 4(b)). An excellent correlation is seen for the conformations and rankings of the low energy conformations between the single point energies and stochastic searches.

Tetraketides

The rapamycin analogue **II** has the same effector domain length as the modified rapamycin ligand RP7 examined in our earlier study.[12] The results for RP7 indicated that the conformational preference of the binding domain might be influenced by the substitutions on the effector domain. The tether is flexible enough that the binding domain can readily assume the binding

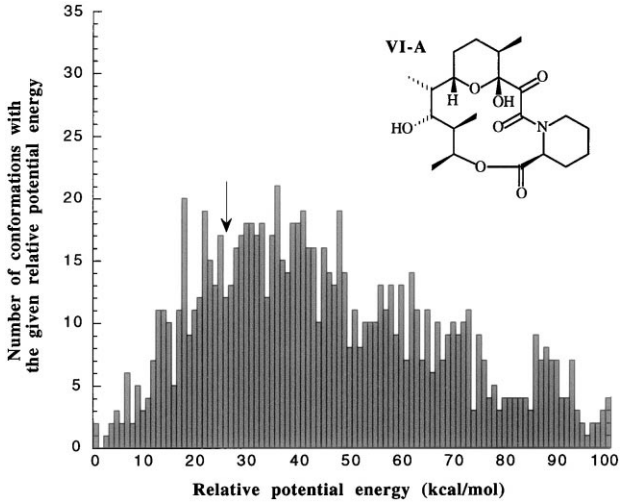


Figure 2. Bar graph showing the location of the lowest energy binding conformation of a tetraketide that does not favor conformations capable of binding to FKBP12. An arrow is used to indicate the location of the lowest energy binding conformation.

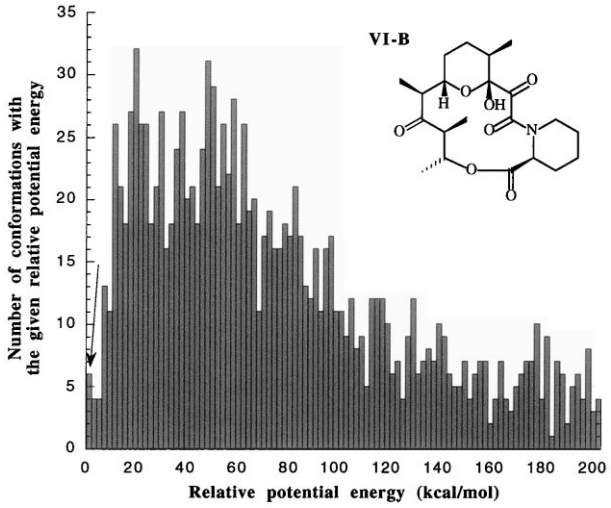


Figure 3. Bar graph showing the location of the lowest energy binding conformation of a tetraketide that favors conformations capable of binding to FKBP12. An arrow is used to indicate the location of the lowest energy binding conformation.

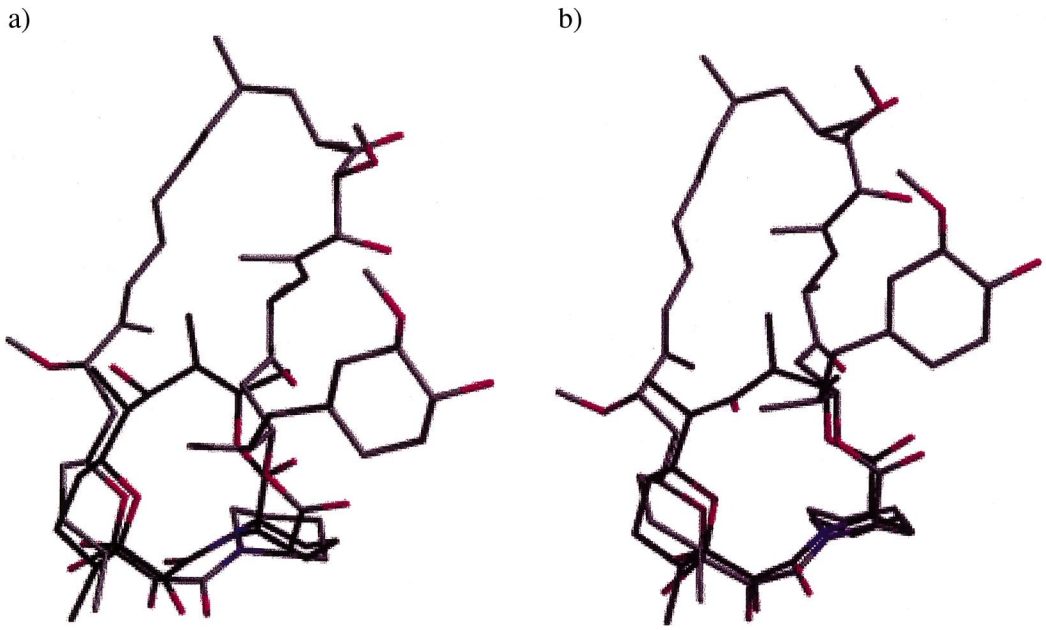


Figure 4. The most favored conformation for each of the molecules in Figures 2 and 3((a) and (b), respectively) overlaid onto the conformation of Rapamycin bound to FKBP12. The preferred conformation of the compound (a) has the opposite orientation of the amide in the binding domain relative to rapamycin.

conformation of rapamycin. The criteria for a suitable binding candidate were, therefore, reduced to 0.3 Å least squares fit between the binding domains of the ligands, rather than the 0.5 Å cut-off applied to the triketide ligands. The stochastic conformational search on **II** revealed a 1.9% probability of residing in any conformation with a better than 0.3 Å least squares fit to the binding domain of rapamycin. The least squares fit between the binding domains is 0.18 Å. **II** was docked in the binding pocket of FKBP12 and a molecular dynamics run was performed on the solvated complex. The average gap between the ligand and FKBP12 was 338 ± 79 Å^3. The SASA of the FKBP12 residues close to the binding pocket was 757 ± 37 Å^2, and the LIE free energy of binding for the complex formation was -3.4 kcal/mol, which is nearly identical (0.1 kcal/mol less favorable) to the binding free energy that was calculated for **I**.

The conformational searches on the 864 tetraketides showed that the substitution patterns can have a marked effect on the conformational preference of the binding domain in much the same way as was observed for the triketide ligands. Bar plots for two representative energy distributions are shown in Figures 5 and 6 (the lowest energy binding structures are indicated by arrows on the figure). The bar plot in Figure 5 is for the tetraketide **V-A**. **V-A** strongly disfavors any of the input

conformations that has a better than 0.5 Å least squares fit to the rapamycin binding domain (a comparison of the lowest energy conformation of **V-A** relative to rapamycin bound to FKBP12 is shown in Figure 7(a)). The bar plot in Figure 6 is for the tetraketide **V-B,** which was predicted by the single point energy calculations to have a 100% probability of existing in a conformation that has a least squares fit of 0.17 Å to the binding conformation of rapamycin (Fig. 7(b)). A stochastic conformational search of **V-B** predicted that the ligand has a 4% probability of existing in the binding conformation. This low probability for the binding conformation comes about because there is another very low energy conformation (roughly 2 kcal/mol lower in energy than any other conformation found). This low energy conformation is characterized by an intramolecular hydrogen bond between the proton on the alcohol substituent and the amide carbonyl oxygen in the binding domain. Such a hydrogen bond would exist in the gas phase but not in aqueous solution. Thus, this conformation was excluded from the probability calculations. When this low energy conformation is omitted, the probability of assuming a suitable binding conformation is 26%. Figure 8 is a stereo view of the favored binding conformation of **V-B**. The substituents along the effector domain are arranged such that no unfavorable steric contacts arise between them, whereas rotations out of the desired binding conformation bring the substituents into close proximity of one another.

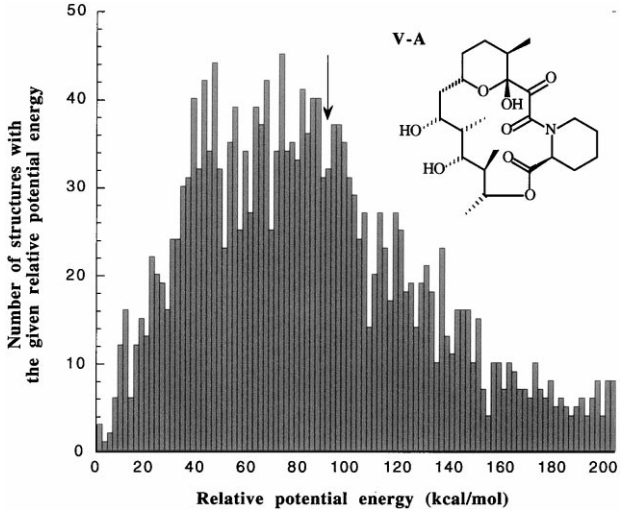


Figure 5. Bar graph showing the location of the lowest energy binding conformation of a pentaketide that does not favor conformations capable of binding to FKBP12. An arrow is used to indicate the location of the lowest energy binding conformation.

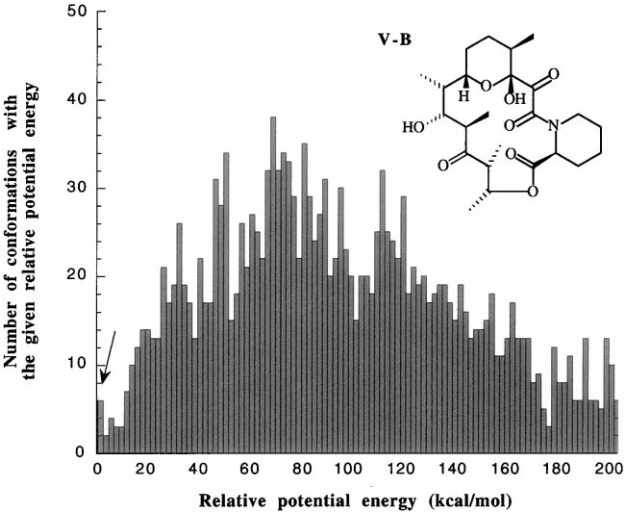


Figure 6. Bar graph showing the location of the lowest energy binding conformation of a pentaketide that favors conformations capable of binding to FKBP12. An arrow is used to indicate the location of the lowest energy binding conformation.

V-B was docked in the binding pocket of FKBP12 and a molecular dynamics run was performed on the complex in solution. The average gap between the ligand and FKBP12 during the MD run is 486 ± 138 Å^3, roughly half the gap that was observed for rapamycin. The SASA of the residues near the binding pocket was 751 ± 38 Å^2. The calculated free energy of binding to FKBP12 was -7.1 kcal/mol, which is 0.5 kcal/mol more favorable than was found for rapamycin and 3.7 kcal/mol more favorable than the interaction energy that was calculated for **II**.

Pentaketides

The template **IV** possesses a tether length equivalent to the modified rapamycin ligand RP6 of our previous study. The results for RP6 did not conclusively indicate whether the substituents on the ligand effector domain had a determining effect on the conformational preference of the binding domain.

The rapamycin analogue **I** has a 0.3% probability of existing in a conformation whose least squares fit to rapamycin is better than 0.3 Å. The lowest energy conformations of **I** have the amide in the opposite configuration from that found in the crystal structure of rapamycin bound to FKBP12. **I** was docked in the binding pocket of FKBP12 and a molecular dynamics simulation was run on the complex in water. The average gap between **I** and FKBP12 during the simulation was 363 ± 88 Å^3, which is slightly smaller than was found for the tetraketide **V-B**. The SASA of the residues near the FKBP12 binding site is 727 ± 83 Å^2, which is nearly identical to the SASA of the rapamycin analogue **III**. This minor effect of the ligand tether on solvent accessibility to the protein comes about because of the rigid nature of the double bond in the tether. The free energy of binding **I** to FKBP12, calculated using the LIE method, was -4.0 kcal/mol, which is 2.6 kcal/mol less favorable than for rapamycin and only 0.5 kcal/mol more favorable than for **III**. Thus, the pentaketide **I** and the triketide **III** are both relatively weak FKBP12 binders based on the LIE and SASA evaluations, showing that the binding affinities to FKBP12 are not correlated to the size of the ligand effector domain.

A conformational study was performed on the 10,386 substitutions for a pentaketide tether. The 349 template conformations examined for the ligands were not biased

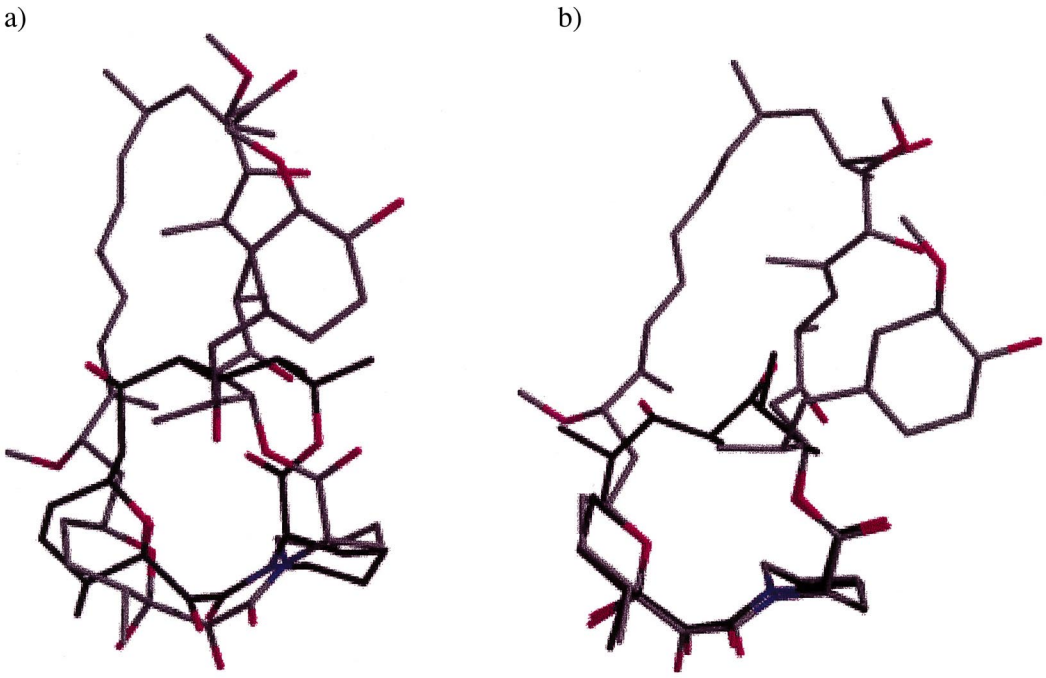


Figure 7. The most favored conformation for each of the molecules in Figures 5 and 6((a) and (b), respectively) overlaid onto the conformation of Rapamycin bound to FKBP12. The preferred conformation of the compound (a) has the opposite orientation of the amide in the binding domain relative to rapamycin.

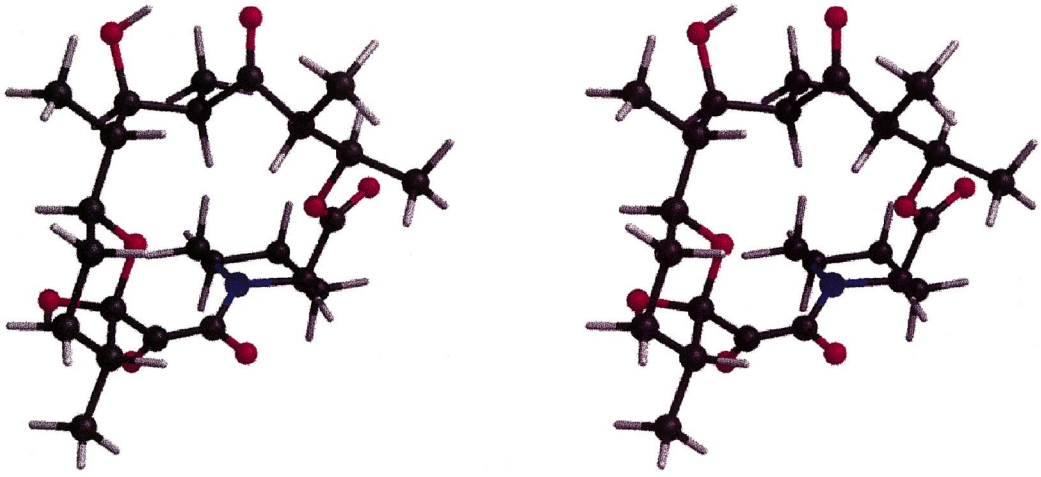


Figure 8. Stereo view of the favored binding conformation of compound **V-B** to FKBP12. The binding domain of the ligand is at the bottom of the picture.

toward any specific orientation and contained roughly even numbers of the two amide conformations. Based on the Boltzmann population distributions of the pentaketides, 995 ligands from the pool were predicted to have over 50% probability of existing in a conformation suitable for binding to FKBP12. The ketides that most frequently appeared at each site in the effector domains of those 995 pentaketides were combined to build the pentaketide **IV-A** shown in Chart 5. **IV-A** was docked in the binding pocket of FKBP12 and an MD simulation was run on the resulting complex.

The average gap between **IV-A** and FKBP12 in the MD simulation was 296 ± 50 Å^3, slightly smaller than was observed for the weaker binder **I**. The average SASA of the residues near the binding pocket was smaller than was observed for **I**, 669 ± 76 Å^2. This was because the preferred conformation of the ligand in the MD simulation was with the tether oriented toward the protein surface. Figure 9 is a stereo view of the favored binding conformation of **IV-A**. As was observed for **V-B**, rotations out of the desired binding conformation bring the substituents on the effector domain into close proximity of one another. The LIE free energy of binding for **IV-A** to FKBP12 was -7.0 kcal/mol, which is 0.4 kcal/mol better than was observed for rapamycin and 3.0 kcal/mol more favorable than the free energy of binding of **I** (Chart 7).

Figure 9. Stereo view of the favored binding conformation of compound **IV-A** to FKBP12. The binding domain of the ligand is at the bottom of the picture.

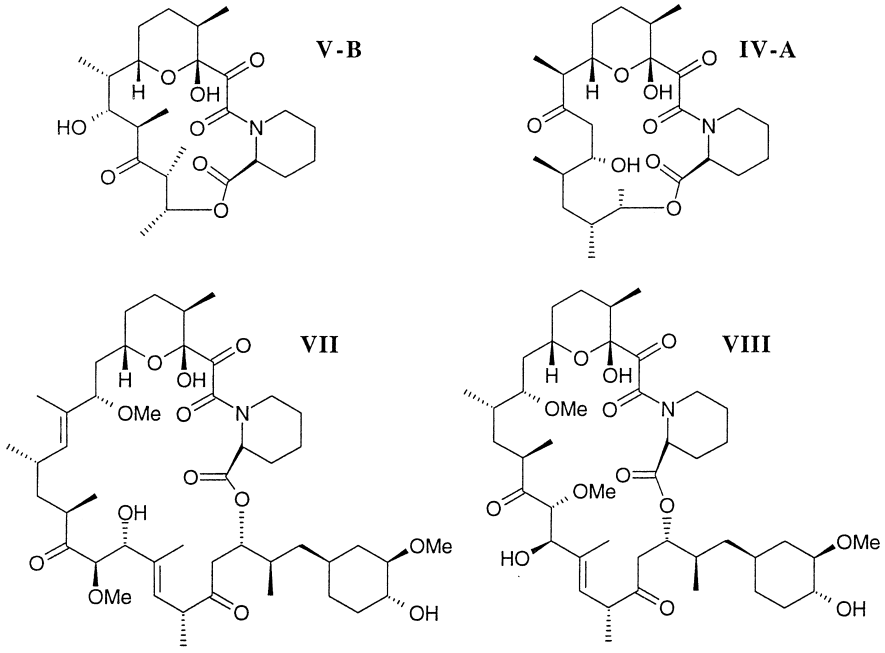


Chart 7. The best FKBP12 binder that emerged from this study and the preceding one. **IV-A** and **V-B** are the results from this study, whereas **VII** and **VIII** are the best binders found by sequential deletions from the effector domain of rapamycin.[12]

Conclusion

In this study and the preceding one, we have used computational methods to design rapamycin analogues with putative neuroregenerative abilities. The design of the molecules presented is such that they are attainable through genetic modifications to the rapamycin polyketide synthase. None of the structures can bind to FRAP and the immunosuppressive activity of rapamycin is, therefore, avoided. The study also reveals that the binding affinity of modified rapamycin analogues to the target protein FKBP12 can be modulated by varying the substituents on the effector domain of the novel polyketide. Good agreement is seen between the probability that a modified rapamycin analogue will exist in the required conformation to bind to FKBP12 and the free energies of binding to the protein target calculated using the LIE method. The study revealed that rapamycin analogues with effector domains corresponding to a tetraketide or pentaketide chain can assume the conformation required to bind to FKBP12, but rapamycin analogues with triketide effector domains are unable to assume the required conformation. The best FKBP12 ligands found using this method have calculated free energies of binding that were comparable to the free energy of binding for rapamycin.

The structures of the four best candidates from the two studies are shown in Chart 5. The first two ligands, structures **V-B** and **IV-A** are the best candidates from the current study. These molecules were designed to be accessible by genetic modifications to the rapamycin

PKS in which units from the effector domain are deleted or replaced. The last two ligands, structures **VII** and **VIII**, were the best candidates from the preceding study. All the ligands in Chart 5 have the same or better predicted binding affinity to FKBP12 as rapamycin.

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