Molecular Mechanics Calculations of Cyclosporin A Analogues. Effect of Chirality and Degree of Substitution on the Side-Chain Conformations of (2*S*,3*R*,4*R*,6*E*)-3-Hydroxy-4-methyl-2-(methylamino)-6-octenoic Acid[1] and Related Derivatives

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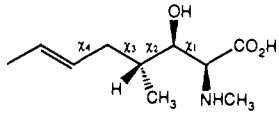
Abstract: Molecular mechanics calculations of cyclosporin (CsA) and three 1-position CsA analogues have been carried out to determine how the chirality and substitution pattern at C4 of analogues of (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid (MeBmt) affect the conformational orientation of this novel amino acid relative to the 33-membered CsA ring system. Systematic conformational searches using the SYBYL software were carried out beginning with the X-ray crystal structure and the deuterochloroform solution conformation of CsA and on the corresponding analogues in which the 1-position residue, MeBmt, had been replaced with the des-C4 methyl analogue (MeBth), the C4 epimer [(4*S*)-MeBmt], and an analogue containing an extra carbon on C4 ($MeBm_2t$). Strategic methodology for implementation of the conformational searches is described. The use of small torsional angle increments (10°) is shown to be essential if all conformational space available to this class of compound is to be sampled. Strategies for selecting conformers prior to minimization are described. Selected conformations were energy minimized by use of the AMBER force field in MacroModel. Boltzmann distributions were calculated for each CsA analogue. Removal of the (4*R*)-methyl group permits the 1-position side chain (MeBth or (4*S*)-MeBmt) to partition into a new conformational family designated XDV. An added methyl group at C4 restricts the conformations accessible to the 1-position side chain ($MeBm_2t$). All four biologically active analogues can access a common conformation. A "bioactive conformation" for immunosuppressive activity is proposed, and the distribution between active and inactive conformers is shown to correlate with immunosuppressive activity.

I. Introduction

Cyclosporin A (CsA), a potent immunosuppressive drug isolated from fungi in 1976 by Dreyfuss et al.,[2] is used clinically to suppress rejection of transplanted human organs.[3] CsA (Figure 1) is a neutral, hydrophobic, cyclic undecapeptide that contains the novel amino acid (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid (**1**; called MeBmt, i.e., *N*-methyl-4-butenyl-4-methylthreonine; formerly called C9-ene) in the 1-position.[4,5] Synthetic analogues in which all 11 amino acids in CsA have been systematically modified have established that MeBmt is essential for immunosuppressive activity.[6-8] Changes in the structure of the MeBmt residue dramatically reduce the immunosuppressive activity of the CsA molecule. Removal of part of the lipophilic side chain of MeBmt, achieved by replacing MeBmt in position 1 with *N*-methylthreonine (**2**) (i.e., $(MeThr)^1CsA$) or with MeLeu(3-OH) (**3**), drastically reduces the compound's immunosuppressive activity.[9] Likewise, removal of the hydroxyl group, as in the derivative containing 3′-desoxy-MeBmt (**4**) results in almost total loss of activity.[10] Thus, a major portion of this novel amino acid appears to be essential for high biological activity.



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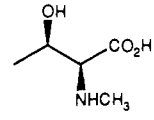

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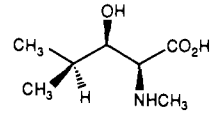

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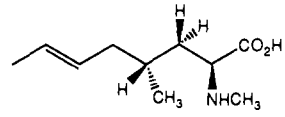

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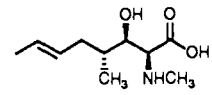
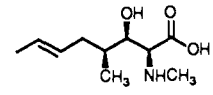
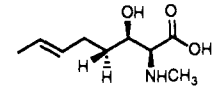
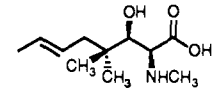
Table I. Structures and Activity of CsA Analogues[a]

amino acid in position 1	abbrevn	CsA analogue	immunosuppressive activity, %
	MeBmt	CsA	100
	(4*S*)-MeBmt	$((4S)\text{-}MeBmt)^1CsA$[b]	2–4
	MeBth	$(MeBth)^1CsA$[b]	10–13
	$MeBm_2t$	$(MeBm_2t)^1CsA$[b]	20–30

[a] Immunosuppressive activities are reported relative to CsA and reflect the degree of inhibition of Concanavalin A stimulated murine thymocytes. [b] Reference 11.

Other side chains in CsA also contribute to immunosuppressive activity. For example, the MeLeu in position 6 has been found

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(1) Abbreviations: CsA, cyclosporin A; MeBmt, (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid; MeBth, (2*S*,3*R*,6*E*)-3-hydroxy-2-(methylamino)-6-octenoic acid; $MeBm_2t$, (2*S*,3*R*,6*E*)-3-hydroxy-4-dimethyl-2-(methylamino)-6-octenoic acid; (4*S*)-MeBmt, (2*S*,3*R*,4*S*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid; XTL, conformational family evolving from a model built from the crystal structure of CsA; SOL, conformational family evolving from a model built from the apolar solution structure of CsA; XDV, conformational family evolving from a model built from the crystal structure of CsA but differing from said model by a rotation of ~120° about χ_2; DMSO, dimethyl sulfoxide; $CDCl_3$, deuterochloroform; IR, infrared; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; RMS, root mean square; CPU, central processing unit.

(2) Dreyfuss, M.; Harri, E.; Hofmann, H.; Kobel, H.; Pache, W.; Tscherter, H. *Eur. J. Appl. Microbiol.* **1976**, *3*, 125.

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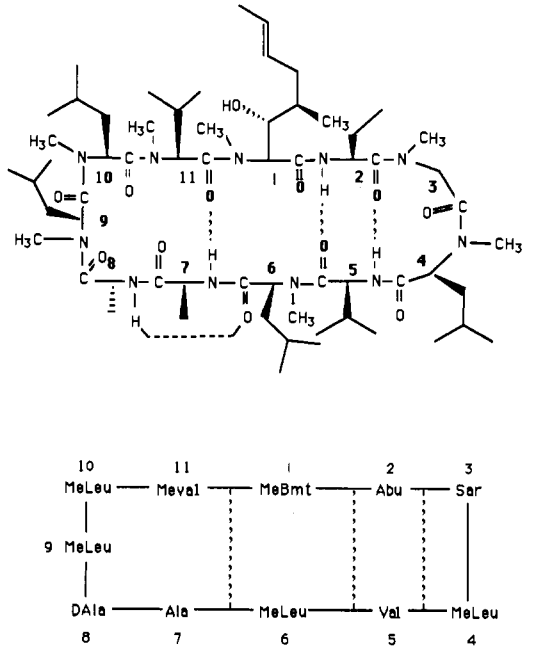

Figure 1. Schematic representation of the primary structure of CsA. Convention used to number the amino acid positions is that proposed by Wenger.[8a] Amide bonds are trans except for MeLeu9–MeLeu10.

to be particularly important because replacement of the isobutyl side chain with a methyl group [as in (MeAla)^{6}CsA] drastically reduces the activity.[11] The three-dimensional interactions between critical side chains on the ring system have yet to be characterized.

The four molecules in Table I illustrate a series of compounds we have prepared in which the MeBmt residue is modified at C4.[12] The (4*S*)-MeBmt and MeBth analogues have only about 2–4% and 10–13%, respectively, of the immunosuppressive activity of the parent structure that contains MeBmt, whereas the more highly substituted $MeBm_2t$ analogue has approximately 20–30% of the biological activity of CsA. Thus, the immunosuppressive capacity of these analogues increases in the following manner: (4*S*)-MeBmt < MeBth < $MeBm_2t$ < MeBmt.

In this paper, we present the results of a theoretical study of CsA analogues that contain MeBth, MeBmt, (4*S*)-MeBmt, and $MeBm_2t$ in the 1-position. The primary objective of this work was to establish the conformations that these compounds might adopt as a function of the chirality and substitution pattern at C4 in MeBmt as a prelude to identifying which conformation(s) of CsA might persist at the receptor site. In addition, this study presents an initial attempt to determine whether there is any correlation between the biological activities of a series of CsA analogues and their side chain conformations.

II. Materials

CsA was obtained from Sandoz. The syntheses of ($MeBm_2t$)^{1}CsA, ((4*S*)-MeBmt)^{1}CsA, and (MeBth)^{1}CsA were carried out as described by Deyo, Sun, and Aebi, respectively.[12] These syntheses closely followed our modifications[12e] of the strategy employed by Wenger[6,7] for the synthesis of CsA and will be reported elsewhere in detail. The biological activities of CsA and the three CsA analogues described above were determined by Dunlap et al. by using the inhibition of concanavalin A stimulated thymocyte assay that has been described previously.[13]

III. Computational Procedure

All molecular modeling was performed on an Evans & Sutherland PS330 with a Micro VAX II serving as the host machine. The crystal structure of native CsA from the Cambridge Crystallographic Data Centre[14] was used as the basis for all subsequent model building carried out with the software package SYBYL, version 5.05.[15]

The conformational analyses were carried out by using the SEARCH subroutine within SYBYL. In all calculations, the SYBYL default van der Waals factors were employed. These multiplicative factors, VDW, serve to reduce the size of the effective van der Waals radii. In the computations described here, VDW = 0.85 for one–four interactions, VDW = 0.65 for hydrogen-bonding interactions, and VDW = 0.95 for all other van der Waals interactions.

Energy minimizations were accomplished using version 1.5 of the software package, MacroModel.[16] This software provides a theoretical method for predicting geometry by means of molecular mechanics (or force field) calculations. The molecular force field utilized in this work was an all-atom version of the AMBER force field,[17] and minimizations were carried out via the Block Diagonal Newton Raphson (BDNR) method.[18] Last, the use of two different molecular modeling software packages required conversion programs for translation of SYBYL and MacroModel coordinate files.[19]

IV. Molecular Models

The structure of native CsA in the solid state has been determined by X-ray crystallography,[20] and, more recently, a solution of CsA in $CDCl_3$ has been elucidated via a molecular dynamics simulation that incorporated 58 distance constraints obtained from IR spectroscopy and NOE data.[21] We elected to use both the crystal and solution structures in our study. The crystal structure of native CsA can be retrieved from the Cambridge Crystallographic Database.[14] Subsequently, the solution structure of CsA was obtained by setting the dihedral angles in both the backbone and the side chains of the crystal structure to the values published

(3) (a) Kahan, B. D., Ed. Proceedings of the First International Congress on Cyclosporin. *Transplant. Proc.* **1983**, *15* (Supplements 1 & 2), 2219–3183. (b) Stiller, C. R.; Keown, P. A. In *Progress in Transplantation*. Morris, P. J., Tilney, N. L., Eds.; Churchill Livingstone: Edinburgh, 1984; Vol. 1, p 11.
(4) Ruegger, A.; Kuhn, M.; Lichti, H.; Loosli, H. R.; Huguenin, R.; Quiquerez, C.; von Wartburg, A. *Helv. Chim. Acta* **1976**, *59*, 1075.
(5) Wenger, R. M. *Helv. Chim. Acta* **1983**, *66*, 2308.
(6) Wenger, R. M. *Helv. Chim. Acta* **1983**, *66*, 2672.
(7) Wenger, R. M. *Helv. Chim. Acta* **1984**, *67*, 502.
(8) (a) Wenger, R. M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 77. (b) Wenger, R. M. *Prog. Chem. Org. Nat. Prod.* **1986**, *50*, 123. (c) Wenger, R. M. *Transplant. Proc.* **1988**, *20*, 313.
(9) Rich, D. H.; Dhaon, M. K.; Dunlap, B.; Miller, S. P. F. *J. Med. Chem.* **1986**, *29*, 978.
(10) Traber, R.; Hofmann, H.; Loosli, H. R.; Ponelle, M.; von Wartburg, A. *Helv. Chim. Acta* **1987**, *70*, 13.
(11) Durette, P. L.; Boger, J.; Dumont, F.; Firestone, R.; Frankshun, R. A.; Koprak, S. L.; Lin, C. S.; Melino, M. R.; Pessolano, A. A.; Pisano, J.; Schmidt, J. A.; Sigal, N. H.; Staruch, M. J.; Witzel, B. E. *Transplant. Proc.* **1988**, *20*, 51.
(12) (a) Sun, C. Q.; Rich, D. H. *Tetrahedron Lett.* **1988**, *29*, 5205. (b) Tung, R.; Dunlap, B.; Aebi, J. D.; Mellon, W.; Ruoho, A. E.; Dhanasekaran, N.; Rich, D. H. In *Synthetic Peptides: Approaches to Biological Problems. UCLA Symposia on Molecular and Cellular Biology, New Series*; Tam, J., Kaiser, T., Eds.; Alan R. Liss, Inc.: New York, 1988; Vol. 86, pp 321–335. (c) Aebi, J. D.; Deyo, D. T.; Guillaume, D.; Rich, D. H., submitted for publication. (d) Rich, D. H.; Sun, C. Q.; Guillaume, D.; Dunlap, B.; Evans, D. A.; Weber, A. E. *J. Med. Chem.* **1989**, *32*, 1982. (e) Aebi, J. D.; Guillaume, D.; Dunlap, B.; Rich, D. H. *J. Med. Chem.* **1988**, *31*, 1805.
(13) Dunlap, B. E.; Dunlap, S. A.; Rich, D. H. *Scand. J. Immunol.* **1984**, *20*, 237.
(14) Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, England.
(15) SYBYL, Molecular modeling system, Tripos Associates, St. Louis, MO.
(16) Still, W. C.; Richards, N. G. J.; Guida, W. C.; Lipton, M.; Liskamp, R.; Chang, G.; Hendrickson, T. *MacroModel V1.5*, Department of Chemistry, Columbia University, New York, NY 10027.
(17) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. *J. Comput. Chem.* **1986**, *7*, 230.
(18) Burkert, U.; Allinger, N. L. *Molecular Mechanics*; ACS Monograph 177, American Chemical Society: Washington, DC, 1982; pp 69–72.
(19) The authors gratefully acknowledge Dr. Michael Czarniecki of Schering Corp. for providing us with FORTRAN routines written by Dr. John Clader that effectively interchange the formats and symbols used in SYBYL and MacroModel coordinate files.
(20) (a) Loosli, H. R.; Kessler, H.; Oschkinat, H.; Weber, H. P.; Petcher, T. J.; Widmer, A. *Helv. Chim. Acta* **1985**, *68*, 682. (b) Petcher, T. J.; Weber, H. P.; Ruegger, A. *Helv. Chim. Acta* **1976**, *59*, 1480.
(21) (a) Lautz, J.; Kessler, H.; Kaptein, R.; van Gunsteren, W. F. *J. Comput.-Aided Mol. Des.* **1987**, *1*, 219. (b) Kessler, H.; Loosli, H. R.; Oschkinat, H. *Helv. Chim. Acta* **1985**, *68*, 661.

Table II. Number of Conformers of CsA Analogues

compd	structure	no. of conformers[a]	reduced no. of conformers[b]	no. of unique conformers[c]
CsA	crystal	400	22	5
	solution	860	46	7
(MeBth)^{1}CsA	crystal	4254	83	14
	solution	1962	92	
(MeBm$_2$t)^{1}CsA	crystal	136	11	2
	solution	305	26	8
((4*S*)-MeBmt)^{1}CsA	crystal	1978	67	13
	solution	126	22	7

[a] Total number of conformers generated by varying four torsional angles in the MeBmt residue (or its derivative) by 10° increments. [b] Number of conformers remaining after data reduction. [c] Number of conformers resulting from minimization of the reduced number of conformers followed by elimination of duplicates.

by Lautz et al. for CsA in the apolar solvent $CDCl_3$.[21a] These structures demonstrate that the peptide has essentially the same backbone conformation both in the crystalline state and in apolar solution (see Figure 2a); however, a significant difference between the two models can be seen in the orientation of the MeBmt side chain (see Figure 2b). As has been noted elsewhere,[21] the MeBmt side chain is folded over the cyclic peptide backbone in the crystal structure. In apolar solution, however, the MeBmt side chain has rotated approximately 120° relative to that in the solid state so that it extends out into the solvent. Also, one should note that, since addition of DMSO to the NMR solvent ($CDCl_3$) results in the formation of multiple conformations,[21b,22] these two structures represent only two of the possible conformations that the CsA molecule might adopt.

Models for all CsA analogues examined here were constructed through appropriate modifications to the crystal and solution structures of CsA described above. For example, by either deleting the methyl group or by adding a second methyl group to C4 of MeBmt, models for the crystal and solution structures were obtained for the MeBth and MeBm$_2$t analogues, respectively. In addition, corresponding models for the (4*S*)-MeBmt derivative were constructed by altering the chirality of the carbon atom at the 4-position of the MeBmt residue in the parent structures. Subsequently, each of these eight structures (i.e., a crystal and solution model for CsA and each of the three analogues) was subjected to an energy minimization.

V. Conformational Analysis

To determine the conformations accessible to the residue in the 1-position of CsA and its analogues, a conformational search was conducted for each of the eight structures described. During this procedure, the torsional angles about the four bonds, χ_1–χ_4 (illustrated in structure **1**) were varied by specified increments and the resulting conformations were examined for van der Waals contacts. (The terminal dihedral angle of the olefin, assumed to be trans, was not varied.) Conformations that allowed two atoms to be closer to one another than the sum of their van der Waals radii were discarded. In this way, the results of a conformational search provide one with ranges of acceptable values for specific dihedral angles and thus the regions of space that the corresponding portions of a molecule might occupy.

A. Strategy Employed during Conformational Search Procedure. Although the primary differences between the solution and crystal structures of CsA and its analogues arise from variations in side chain orientations (most notably the MeBmt^1 and MeLeu^{10} residues), subtle variations in the backbone conformations proved to be important during the conformational analyses. For example, a conformational search of the 1-position residue of the crystal structure of CsA (folded orientation of the MeBmt side chain) yields a variety of conformers that all retain the folded orientation of MeBmt. The extended orientation of MeBmt ($CDCl_3$ model of CsA) does not result from a conformational search of the CsA crystal structure due to bad van der Waals interactions between the MeBmt^1 and MeLeu^6 side chains. Even allowing the MeLeu^6 side chain to rotate (two additional rotatable bonds) did not relieve these unfavorable van der Waals interactions so that it could access the orientation that it assumes in $CDCl_3$. These results indicate that rotation of the MeBmt side chain from the folded to the extended position must be accompanied by a simultaneous, yet subtle, movement of the backbone structure.

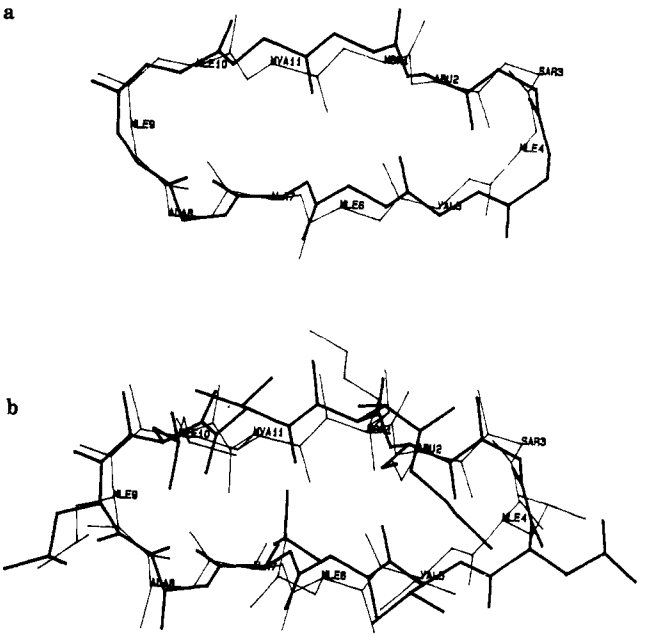

Figure 2. (a) Superposition of the peptide backbone of the crystal and solution structures of CsA. The crystal structure is depicted by thick lines and the solution structure by thin lines. (b) Superposition of the crystal and solution structures of CsA with side chains but minus the hydrogen atoms. The crystal structure is depicted by thick lines and the solution structure by thin lines. Note the difference in the orientation of the MeBmt side chain in the two structures.

Similarly, a conformational search of the MeBmt side chain in the apolar solution structure of CsA (extended orientation of MeBmt) does not reveal any conformers that can accommodate a folded MeBmt side chain. In this case, the folded orientation of the MeBmt side chain is rejected due to negative van der Waals interactions between the MeBmt^1 and MeLeu^4 side chains, which are not relieved when the positioning of the MeLeu^4 side chain is systematically altered. Again, we conclude from these results that a retraction of the MeBmt side chain from the extended orientation requires a concurrent change in the cyclic peptide backbone conformation. Therefore, in order to systematically evaluate all possible conformations of the MeBmt residue in CsA, we would need to incorporate the mobility of the peptide backbone within our calculations. However, to carry out a conformational search of this nature would require defining a ring-closure bond[23] in the backbone ring and several additional rotatable bonds in the backbone and, perhaps, some of the side chains of CsA. Since

(22) Guillaume, D., unpublished data.

(23) A ring-closure bond is a bond that is effectively removed or "broken" during a conformational search, allowing torsional angles in the ring to rotate freely.

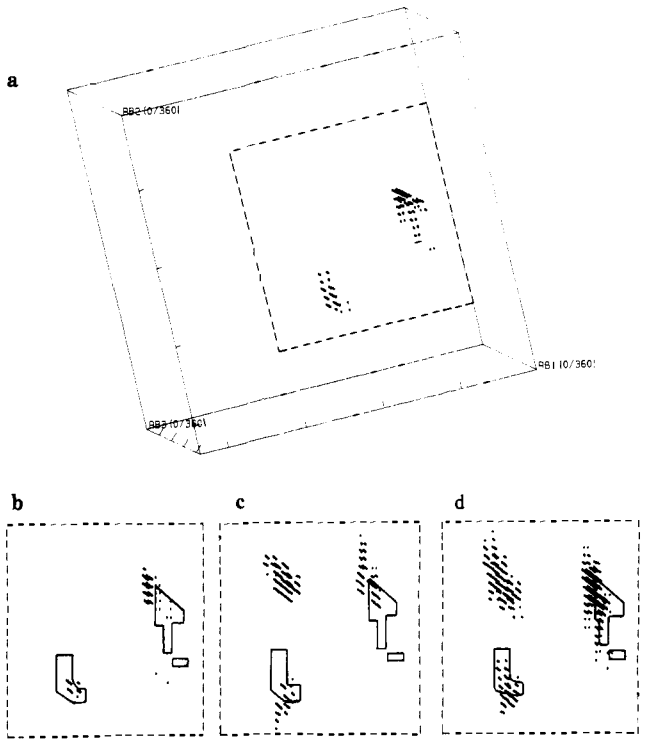

Figure 3. (a) Superposition of the angle maps for the crystal and solution structures of CsA. The values of the torsional angles, χ_1–χ_3, in the MeBmt side chain are plotted along the x, y, and z axes, respectively. The dimensions of the angle maps are 360° × 360° × 360°. (b) Superposition of a region of the angle maps (illustrated by dashed lines in Figure 3a) for the crystal and solution structures of $(MeBm_2t)^1$CsA. Templates illustrate the regions of occupancy of the corresponding angle maps for CsA. (c) Superposition of a region of the angle maps (illustrated by dashed lines in Figure 3a) for the crystal and solution structures of $(MeBth)^1$CsA. Templates illustrate the regions of occupancy of the corresponding angle maps for CsA. (d) Superposition of a region of the angle maps (illustrated by dashed lines in Figure 3a) for the crystal and solution structures of $((4S)\text{-MeBmt})^1$CsA. Templates illustrate the regions of occupancy of the corresponding angle maps for CsA.

the central processing unit (CPU) time required for a conformational search increases dramatically with the number of rotatable bonds, we elected to use the alternative strategic approach described (vide supra) toward resolving the various conformations accessible to the 1-position residue of CsA and its analogues.

B. Use of Angle Maps To Interpret the Conformational Space Available to Each Analogue. The number of viable conformations of CsA and its analogues generated during the conformational search procedure are presented in Table II. Because the amount of data generated in a search is often overwhelming (i.e., a tabulation of the torsional angle values for each viable conformation), we chose to visualize the results through the use of angle maps.[24] For example, a superposition of the angle maps resulting from a conformational search of the crystal and solution structures of CsA is depicted in Figure 3a. In this illustration, the values of the torsional angles χ_1, χ_2, and χ_3 are plotted along the x, y, and z axes, respectively. Each dot in this figure represents one or more conformers with the corresponding values for χ_1, χ_2, and χ_3. In general, a cluster of dots illustrates a "conformational family" or a dense region of angle space that the MeBmt side chain may occupy.

From the angle maps shown in Figure 3a, one can see that there are essentially two families of conformers arising from the torsional angles χ_1, χ_2, and χ_3 accessible to the CsA molecule. These two familes have approximately the same range of values for the torsional angles χ_3 and χ_4 but very different values for χ_1 and χ_2. In one conformational family, the torsional angle χ_1 can assume values ranging from about 190–200° while the range for χ_2 is approximately 80–120°. In contrast, the second family of conformers has values of χ_1 and χ_2 that span from about 315–320° and 130–190°, respectively. Note that χ_1 differs by approximately 120° between these two families of conformers. Indeed, the crystal structure of CsA (where χ_1 is approximately 191°) is a member of the first conformational family, XTL, while the apolar solution structure (with $\chi_1 = \sim 316°$) belongs to the second conformational family, SOL.

Figure 3b depicts a superposition of the angle maps for the $MeBm_2t$ analogue. As in the previous case, Figure 3b illustrates that there are only two primary families of conformers that are accessible to $(MeBm_2t)^1$CsA based on variations in χ_1, χ_2, and χ_3. Moreover, since the $MeBm_2t$ analogue is more highly substituted than CsA, one might expect that it will be more conformationally constrained as well. This conclusion is supported by the fact that the two clusters of dots corresponding to the conformational families of $(MeBm_2t)^1$CsA are essentially subsets of those of CsA.

A superposition of the angle maps for $(MeBth)^1$CsA is illustrated in Figure 3c. Interestingly, three viable conformational families correspond to differing values of χ_1, χ_2, and χ_3 in $(MeBth)^1$CsA. Two of these families contain the crystal and solution structures, respectively, while the third family of conformers, XDV, arises from a rotation of approximately 120° about χ_2 in the crystal structure. These results are more clearly delineated in Table III. In addition, since the two families of conformers representing CsA are subsets of the corresponding $(MeBth)^1$CsA conformational families, CsA is more conformationally constrained than the MeBth analogue.

Last, the angle maps for the (4*S*)-MeBmt derivative are presented in Figure 3d. This figure shows that, similar to the latter analogue, there are three families of conformers accessible to χ_1, χ_2, and χ_3 of $((4S)\text{-MeBmt})^1$CsA. In fact, these three conformational families occupy the same, albeit smaller, regions of angle space as those representing the MeBth analogue.

Table III. 1-Position Torsional Angle Ranges for $(MeBth)^1$CsA Structures[a]

	XTL	SOL	XDV
χ_1	190–200	310–315	195–210
χ_2	60–105	120–240	180–255
χ_3	75–210	75–300	45–300
χ_4	45–315	45–315	45–315

[a] Torsional angle ranges for the three conformational families resulting from varying four torsional angles in the MeBth residue of the crystal and solution structures of $(MeBth)^1$CsA by 10° increments.

VI. Energy Minimizations

The search algorithm, which functions solely on the basis of steric (or van der Waals) interactions, produced a multitude of conformations accessible to the 1-position side chain of CsA and its analogues (see column 3 in Table II). In order to determine the optimal (or most stable) conformations of these compounds, each viable conformer should be refined via energy minimization. Each low-energy structure resulting from energy minimization represents a local minimum on the potential energy surface, and, in many cases, two different conformers will converge to the same local minimum after minimization, thereby eliminating duplicate structures. Therefore, by systematically exploring different sets of starting coordinates for each molecule (i.e., all viable conformers), we should be able to locate the global minimum, as well as all local minima, which correspond to relatively stable orientations of the 1-position side chain. Any of these structures must be regarded as a potential source of biological activity, since there is no reason, a priori, to exclude high-energy conformations.

A. Data Reduction. In this study, the conformers resulting from the conformational searches performed on CsA and its analogues were used as starting points for energy minimizations. In theory, we would like to minimize each conformer generated. Unfortunately, the searches performed here produced a number of conformations that were too large for facile analysis given our

(24) Mayer, D.; Naylor, C. B.; Motoc, I.; Marshall, G. R. *J. Comput.-Aided Mol. Des.* **1987**, *1*, 3 and references cited therein.

Table IV. Torsional Angle Ranges Obtained from a Conformational Search of the Crystal Structure of CsA[a]

χ_1	χ_2	χ_3	χ_4
190–200	80–120	80	90–110
190–200	80–120	120–130	90–120
190–200	80–120	120–130	240–270
190–200	80–120	170–220	50–280

[a] Four torsional angles in the MeBmt residue were varied by 10° increments.

computational resources. So in order to analyze the structures resulting from the conformational searches, it was necessary to develop a technique that would permit us to sample the results. Specifically, we chose to employ a nonrandom means of decreasing the number of conformers to undergo energy minimization.

Table IV presents the possible ranges of values for the dihedral angles χ_1–χ_4 in the crystal structure of CsA that were generated during a conformational search employing 10° increments. A total of 400 viable conformers emerged from this particular search. In an attempt to reduce the number of conformers requiring minimization, the search procedure was repeated using a 30° increment for χ_2, χ_3, and χ_4 and a 20° increment for χ_1 (larger angle increments for χ_1 yielded zero viable conformers). These larger angle increments produced seven viable conformers. However, as one can see from the data in Table IV, a 30° increment for χ_3 and χ_4 misses significant regions of space that are accessible to the molecule. These neglected regions were examined by additional conformational searches that employed smaller angle increments for χ_3 and χ_4. The resulting conformers were subsequently examined, and, for cases in which a continuous range of values resulted for a particular dihedral angle (values of all other dihedral angles being constant), all members that differed from a selected member by less than 30° were deleted. In this manner, 15 conformers were added to the seven conformers generated previously. These 22 conformers originating from the crystal structure of CsA were subjected to energy minimization. In an analogous fashion, the number of conformers evolving from the solution structure of CsA, and both the crystal and solution structures of the three CsA analogues, was systematically decreased (see Table II).

B. Convergence Criteria and Elimination of Duplicate Structures. A series of preliminary energy minimizations was performed on the reduced number of viable conformers for each structure, using a criterion that stipulated a gradient (first derivative RMS) convergence of 0.01 kJ·mol^{-1}·Å^{-1} (or 2.39 × 10^{-3} kcal·mol^{-1}·Å^{-1}).[25] The objective of these somewhat lenient minimizations was to eliminate duplicate structures, and the results are illustrated in the last column of Table II. Subsequently, all unique structures were subjected to a more stringent energy minimization that utilized a gradient convergence of 0.001 kJ·mol^{-1}·A^{-1} (or 2.39 × 10^{-4} kcal·mol^{-1}·A^{-1}). The results of these calculations are presented in Tables V–VIII. In addition, the specific contributions to the total energy of the minimum energy conformer arising from each conformational family for each CsA analogue are reported in Tables X–XIII in the supplementary material. The energy minimizations for CsA required a total of 21 days of CPU time on a Micro VAX II, and those for the MeBm$_2$t and (4*S*)-MeBmt analogues took approximately 9 and 31 CPU days, respectively. Minimization of the conformers derived from the crystal structure of (MeBth)^{1}CsA required 42 CPU days.

C. Population Analysis. In order to determine the relative populations of the various conformations of each CsA analogue, a series of population analyses have been performed. By applying the Boltzmann distribution

$$P_i = P_j\{\exp[-(E_i - E_j)/RT]\}$$

to the set of conformations accessible to a particular molecular system, one can calculate the proportion of molecules in each conformation i with energy E_i at temperature T (assuming the system is in thermal equilibrium). Assuming that the number of unique conformations for each analogue reported in Table II represents all conformations accessible to the 1-position residue in these systems, it is then possible to predict the distribution of a molecular system over a set of conformations or, in other words, the percentage of time that a given compound spends in each accessible conformation.

D. Effect of Population on Drug–Receptor Apparent Dissociation Constant. At each instant, only a fraction of the molecules exposed to a receptor will have the proper conformation for binding to the receptor to produce a biological response. We define these conformations as the "bioactive conformer(s)" of cyclosporin. Although the concept of bioactive conformers has a long history,[40] the relationship of the bioactive conformers to the nonbioactive conformers has not been explicitly related to the biological response. It can be seen for a system with n inactive conformers

$$\{C^I\}_i \qquad i = 1, 2, ..., n$$

in equilibrium with the biologically active conformer(s), C^A

$$\{C^A\} \rightleftarrows \{C^I\}$$

that

$$K_{eq} = [C^I]/[C^A] \tag{1}$$

If we assume that the inactive conformers (C^I) have little affinity for the receptor relative to the active conformer (C^A), then the *intrinsic dissociation constant*, i.e., the dissociation constant for the receptor and the bioactive conformer, can be defined as eq 2. The observed dissociation constant for all the CsA molecules is related to the intrinsic dissociation constant by eq 3. The biological activity of each analogue relative to CsA can be expressed as the ratio in eq 4. If K_d^{int} for each analogue is the same

$$K_d^{int} = [C^A][R]/[C^AR] \tag{2}$$

$$K_d^{obs} = K_d^{int}[1 + K_{eq}] \tag{3}$$

$$\text{rel activity (\%)} = \frac{[K_d^{int}(1 + K_{eq})]_{CsA}}{[K_d^{int}(1 + K_{eq})]_{analogue}} \times 100 \tag{4}$$

as for CsA, then the relative activity of each analogue is reduced to an expression in terms of the concentration of the bioactive conformers:

$$\text{rel activity (\%)} = \frac{(1 + K_{eq})_{CsA}}{(1 + K_{eq})_{analogue}} \times 100 \frac{[C^A]_{analogue}}{[C^A]_{CsA}} \times 100 \tag{5}$$

VII. Results

In this section, we describe the various conformations that the 1-position residue of CsA and its analogues may adopt. In doing so, we will attempt to assess the consequences of altering the chirality and substitution pattern of C4 in MeBmt.

A. CsA. As Table V illustrates, a total of 12 conformations of CsA have been identified (see Figure 5 in the supplementary material) and, because all are within 4 kcal/mol of the minimum energy conformer, none of these conformers can be eliminated from consideration on the basis of energetics alone. It is of interest that the calculations indicate that the conformers originating from the crystal structure of CsA are clearly lower in energy than those derived from the structure of CsA in $CDCl_3$. In fact, the population analysis predicts that the five conformers obtained from the crystal structure of CsA comprise 99% of the equilibrium mixture. However, NMR data obtained in $CDCl_3$ show that one conformer of CsA predominates and the coupling constant ($[^3J_{HC^\alpha C^\beta H}]^1$ = 5.7 Hz)[20a] corresponds to a χ_1 value for the MeBmt side chain of about 317–319°.[26] Thus, in contrast to the computational results, the NMR data imply that there is a stabilizing

(25) (a) For a discussion of optimization criteria, see: Reference 18, p 61. (b) For a discussion of gradient convergence criteria, see: MacroModel[14] documentation, "MMTPL.DOC".

(26) Bystrov, V. F. *Progr. NMR Spectrosc.* **1976**, *10*, 41.

Table V. Relative Energies, Optimized Torsional Angles, and Relative Populations of the CsA Conformers Determined from Molecular Mechanics Calculations[a]

conformer	conformational family	E_{rel}[b]	χ_1	χ_2	χ_3	χ_4	%[c]
A	XTL	0.000	191.0	74.2	184.9	196.5	34.617
B	XTL	0.099	194.4	81.7	194.1	221.6	29.290
C	XTL	0.167	178.7	52.9	59.4	112.4	26.114
D	XTL	0.926	196.0	77.5	180.0	79.5	7.253
E	XTL	1.867	193.1	83.6	86.0	253.4	1.482
F	SOL	2.562	291.9	156.1	57.3	178.8	0.458
G	SOL	2.719	317.3	180.0	61.2	98.1	0.352
H	SOL	3.015	316.3	185.3	68.9	163.0	0.213
I	SOL	3.641	316.0	183.9	182.0	68.4	0.074
J	SOL	3.879	315.1	188.3	287.1	165.3	0.050
K	SOL	3.885	314.5	188.5	288.6	286.5	0.049
L	SOL	3.915	315.6	186.1	184.2	250.2	0.047

[a] Relative energies are in kilocalories per mole, torsional angles are in degrees, and relative populations are reported as the equilibrium percentage assuming only 12 conformations of CsA and T = 25 °C. [b] All energies are relative to conformer A; the lowest energy conformer. [c] Population distribution.

Table VI. Relative Energies, Optimized Torsional Angles, and Relative Populations of the $(MeBm_2t)^1$CsA Conformers Determined from Molecular Mechanics Calculations[a]

conformer	conformational family	E_{rel}[b]	χ_1	χ_2	χ_3	χ_4	%[c]
A	XTL	0.000	198.3	69.0	176.6	93.2	77.396
B	XTL	0.732	198.1	69.0	183.8	258.0	22.498
C	SOL	4.803	303.7	198.1	292.3	263.9	0.023
D	SOL	4.837	303.1	191.5	174.0	260.6	0.022
E	SOL	5.108	304.4	196.1	288.5	182.6	0.014
F	SOL	5.152	301.9	187.9	66.4	99.6	0.013
G	SOL	5.178	303.2	192.5	176.3	100.7	0.012
H	SOL	5.436	307.0	192.0	72.4	159.5	0.008
I	SOL	5.549	290.4	193.4	289.0	108.1	0.007
J	SOL	5.615	303.3	190.9	281.9	106.9	0.006

[a] Relative energies in kilocalories per mole, torsional angles are in degrees, and relative populations are reported as the equilibrium percentage assuming only 10 conformations of $(MeBm_2t)^1$CsA and T = 25 °C. [b] All energies are relative to conformer A; the lowest energy conformer. [c] Population distribution.

Table VII. Relative Energies, Optimized Torsional Angles, and Relative Populations of the ((4S)-MeBmt)^{1}CsA Conformers Determined from Molecular Mechanics Calculations[a]

conformer	conformational family	E_{rel}[b]	χ_1	χ_2	χ_3	χ_4	%[c]
A	XDV	0.000	193.6	201.8	68.3	178.5	25.418
B	XDV	0.353	192.6	198.1	64.2	249.2	14.008
C	XDV	0.514	180.0	181.9	163.2	277.1	10.675
D	XTL	0.544	197.3	64.8	171.2	100.8	10.148
E	XDV	0.619	184.2	194.7	62.7	68.1	8.941
F	XTL	0.668	185.7	46.6	168.3	231.4	8.232
G	XDV	0.790	194.5	207.5	297.8	99.3	6.700
H	XDV	0.848	181.9	180.0	167.0	166.1	6.075
I	XDV	1.176	194.5	208.6	301.8	267.8	3.492
J	XDV	1.366	178.7	180.9	163.0	279.5	2.534
K	XDV	1.447	181.4	180.0	167.0	164.6	2.210
L	SOL	2.179	301.0	194.3	167.2	270.0	0.643
M	SOL	2.916	306.3	230.2	290.8	204.3	0.185
N	SOL	3.051	302.5	205.6	292.7	257.8	0.147
O	SOL	3.079	301.7	198.5	169.7	107.8	0.141
P	SOL	3.102	301.9	199.3	169.7	164.4	0.135
Q	XTL	3.215	198.7	73.1	74.5	77.2	0.112
R	SOL	3.225	302.4	202.9	287.3	186.9	0.110
S	XTL	3.512	198.5	72.1	80.9	160.2	0.068
T	SOL	4.095	302.2	198.7	279.8	96.6	0.025

[a] Relative energies are in kilocalories per mole, torsional angles are in degrees, and relative populations are reported as the equilibrium percentage assuming only 20 conformations of ((4S)-MeBmt)^{1}CsA and T = 25 °C. [b] All energies are relative to conformer A; the lowest energy conformer. [c] Population distribution.

interaction in deuterochloroform that favors the extended orientation of the MeBmt side chain. Since the calculations were carried out in vacuo (i.e., without any solvent structure), interactions between the CsA molecule and the $CDCl_3$ solution have been neglected. In a recent study, van Gunsteren et al. have shown that molecular dynamics simulations performed in the absence of solvent tend to predict that the most stable conformations of a molecule are those that maximize the intramolecular van der Waals interactions.[27] Similarly, force field calculations performed in vacuo favor conformations in which the side chains of a peptide are folded toward the backbone, thereby enhancing van der Waals interactions.

From Table X (supplementary material) it is apparent that, in the low-energy solution structure, there is more strain arising from bond-stretching, angle-bending, torsional, and van der Waals

(27) Lautz, J.; Kessler, H.; van Gunsteren, W. F.; Weber, H. P.; Wenger, R. M., submitted for publication.

Table VIII. Relative Energies, Optimized Torsional Angles, and Relative Populations of the (MeBth)^{1}CsA Conformers[a] Determined from Molecular Mechanics Calculations[b]

conformer	conformational family	E_{rel}[c]	χ_1	χ_2	χ_3	χ_4	%[d]
A	XDV	0.000	199.3	185.7	180.0	74.5	40.985
B	XTL	0.327	187.8	60.9	168.1	201.5	23.601
C	XDV	1.014	209.5	184.9	190.6	195.7	7.402
D	XDV	1.177	204.1	181.7	64.1	186.0	5.622
E	XTL	1.318	195.3	78.2	181.5	289.8	4.431
F	XTL	1.402	178.7	51.8	59.9	112.0	3.845
G	XDV	1.569	193.5	177.1	54.3	69.2	2.901
H	XDV	1.584	203.6	180.0	59.8	250.0	2.828
I	XDV	1.595	196.2	183.8	285.9	294.4	2.776
J	XTL	1.762	190.0	54.7	157.5	35.7	2.094
K	XTL	1.840	195.3	74.0	174.2	91.3	1.836
L	XDV	2.242	198.7	193.0	291.3	115.2	0.932
M	XDV	2.527	205.0	282.8	293.8	119.6	0.576
N	XTL	3.241	194.0	86.8	86.8	245.9	0.173

[a] Conformers resulting from a conformational search of the (MeBth)^{1}CsA model derived from the crystal structure of CsA. [b] Relative energies are in kilocalories per moles, torsional angles are in degrees, and relative populations are reported as the equilibrium percentage assuming only 14 conformations of (MeBth)^{1}CsA and T = 25 °C. [c] All energies are relative to conformer A; the lowest energy conformer. [d] Population distribution.

interactions than in the low-energy crystal structure. However, the contributions resulting from electrostatic and hydrogen-bonding interactions are more stabilizing in the former than in the latter conformer. This stabilization in conformers with the extended orientation of the MeBmt side chain almost certainly arises from the hydrogen bond that is formed between the hydroxyl hydrogen on the MeBmt side chain and the carbonyl oxygen in the MeBmt backbone. In contrast, in conformers arising from the crystal structure of CsA, the MeBmt hydroxyl group does not participate in any intramolecular hydrogen bonds. Notably, the same qualitative trends in the components of the potential energy of conformers with the extended vs folded orientation of the 1-position side chain are apparent in the CsA analogues considered here (e.g., see Tables XI and XII in the supplementary material).

The MeBmt side-chain torsional angle values for the conformations of CsA are presented in Table V. Note that conformers A and B of CsA are extremely similar; their respective values for χ_1, χ_2, and χ_3 are within 10° of each other while their χ_4 values differ by about 25°. Since these two conformers of CsA correlate more closely with each other than with any of the XTL conformers of the CsA analogues and since they only differ in energy by about 0.1 kcal/mol, for our purposes we will not distinguish between them. In general, the conformers arising from the X-ray crystal structure (i.e., the XTL conformers) and those arising from the apolar solution structure (i.e., the SOL conformers) of CsA have distinctly different values for χ_1 and χ_2 yet similar angle ranges for χ_3 and χ_4. In both sets of conformers, the tail end of the MeBmt side chain has a much higher degree of flexibility than does the base connected to the backbone.

B. (MeBm$_2$t)^{1}CsA. Conformational searches and subsequent energy minimizations have identified 10 unique conformers of (MeBm$_2$t)^{1}CsA that are displayed in Table VI (and Figure 6 in the supplementary material). Two low-energy conformers originate from the crystal structure, while eight conformations from the solution structure of (MeBm$_2$t)^{1}CsA are of substantially higher energy. Note that the minimum energy solution conformer of (MeBm$_2$t)^{1}CsA is 4.8 kcal/mol higher in energy than the corresponding minimum energy crystalline conformer. In contrast, the corresponding minimum energy conformers for the parent structure, CsA, differ by only 2.6 kcal/mol. Thus, the more conformationally constrained (MeBm$_2$t)^{1}CsA cannot accommodate the extended conformation of the 1-position side chain as favorably as CsA. Specifically, the solution structures of (MeBm$_2$t)^{1}CsA are more destabilized by bond-stretching, angle-bending, and improper torsional interactions than are the SOL conformers of CsA (compare the last columns in Tables X and XI in the supplementary material). Similar to the CsA molecule, the population analysis of (MeBm$_2$t)^{1}CsA indicates that the XTL conformers comprise over 99% of the equilibrium mixture.

As one might expect, since (MeBm$_2$t)^{1}CsA is more highly substituted than CsA, there are only two conformers accessible to the crystal structure of (MeBm$_2$t)^{1}CsA whereas five originate from CsA. Morever, Table VI shows that the two XTL conformers of (MeBm$_2$t)^{1}CsA are extremely similar, differing primarily in the value of the torsional angle χ_4. Thus, the presence of the extra methyl group at C4 of the MeBm$_2$t side chain severely restricts its movement. Significantly, the torsional angle χ_3 in the XTL conformers of (MeBm$_2$t)^{1}CsA is essentially locked in the trans position.

Last, the results in Table VI indicate that the solution structure of (MeBm$_2$t)^{1}CsA does not appear to be significantly influenced by the presence of the additional methyl group on the 1-position side chain. Seven conformations are accessible to the solution structure of CsA whereas there are eight SOL conformers of (MeBm$_2$t)^{1}CsA. As in CsA, the (MeBm$_2$t)^{1}CsA SOL conformers have extremely similar values of χ_1 and χ_2 and very different values of χ_3 and χ_4.

C. [(4*S*)-MeBmt]^{1}CsA. The 20 conformations accessible to the 1-position side chain of ((4*S*)-MeBmt)^{1}CsA are presented in Table VII. Thirteen conformers originate from the crystal structure while only seven conformers evolve from the solution structure (Figure 7 in the supplementary material). Compared to the results for CsA and (MeBm$_2$t)^{1}CsA, the data in Table VII indicate that the absence of a (4*R*)-methyl group on the 1-position side chain results in a significantly higher degree of mobility in the crystal structure of the (4*S*)-MeBmt analogue, while the corresponding solution structure retains comparable flexibility to those described previously. Only four of the crystallike conformers of ((4*S*)-MeBmt)^{1}CsA (i.e., conformers D, F, Q, and S) correspond to those identified for CsA and (MeBm$_2$t)^{1}CsA.

The remaining nine conformers that arise from the crystal structure of ((4*S*)-MeBmt)^{1}CsA are geometrically different from any of the conformers we have considered thus far. These conformers belong to a different conformational family, XDV, in which χ_2 has an average value of 193° that differs from that for the XTL conformers by approximately 128°. However, the geometrical differences between the XTL and XDV conformers are especially apparent when one considers the orientation of the (4*S*)-methyl group with respect to the (3*R*)-hydroxyl moiety on the MeBmt side chain. The dihedral angle, HO–C3–C4–Me, in the XTL conformers has an average value of 174° while that in the XDV conformers has an average value of 317°.

In comparison to the minimum energy XTL structure of ((4*S*)-MeBmt)^{1}CsA (conformer D), the minimum energy XDV structure (conformer A) appears to be more stable energetically due to favorable bond-stretching, angle-bending, and electrostatic interactions (see Table XII in the supplementary material). While conformer D has less strain than conformer A arising from torsional and van der Waals interactions, these are not sufficient to compensate for the destabilizing interactions listed above. In accordance with these data, the population analysis for ((4*S*)-MeBmt)^{1}CsA predicts that the equilibrium mixture will contain

80.0% XDV conformers, 18.6% XTL conformers, and only 1.4% SOL conformers.

D. (MeBth)^{1}CsA. As illustrated in Table VIII, a conformational search and subsequent energy minimizations characterized 14-conformations accessible to the MeBth side chain in the model representing the (MeBth)^{1}CsA crystal structure (see Figure 8 in the supplementary material). However, energy minimizations for the corresponding SOL conformers were not carried out due to limited computational resources. Of the 14 MeBth conformers that have been identified, eight belong to the XDV conformational family and six are members of the XTL conformational family. In contrast to [(4*S*)-MeBmt]^{1}CsA, for which the ratio of XTL to XDV conformers is 23:100, the corresponding ratio of (MeBth)^{1}CsA conformers is 56:100. Therefore, a population analysis of crystalline (MeBth)^{1}CsA indicates that the XDV and XTL conformers comprise 64% and 36% of the equilibrium mixture, respectively.

VIII. Discussion

A. Strategic Methodology. The primary theoretical techniques employed in this study are generation of molecular conformers and subsequent refinement of conformers via energy minimization. The latter technique is crucial to determining the relative contribution of different conformers to the equilibrium properties of a molecule. Although the theory on which these techniques are based is fairly straightforward, there is no standard recipe for their application. Rather, the implementation of molecular modeling techniques remains a creative process that will necessarily depend upon the investigator and the project under consideration. Therefore, features unique to this study include the methodological strategies that have been developed. During the course of studying the conformational features of a series of CsA analogues, we repeatedly found that conventional methods of implementing standard theoretical tools were either intractable or subject to major caveats. These difficulties were resolved via the approaches described below.

Computational Setup. A first step in the CsA project was to carry out a series of conformational searches. Although a variety of methods exist for generating conformational surfaces,[28] we chose to utilize a systematic search method (i.e., a grid search). In the discussion that follows, we describe the conformational searching strategy that we employed and we emphasize the importance of determining optimal dihedral angle increments.

The task of performing a systematic conformational search of the 1-position side chain of the CsA molecule forced us to devise a unique method of applying the search function in SYBYL. As described in section V.A, a rigorous search of the MeBmt side-chain conformations of the crystal structure of CsA does not produce any conformations in which MeBmt is in an extended (solution) conformation. However, we know that stable conformations of CsA exist in which the MeBmt side chain extends into solvent (section IV). The extended orientation of MeBmt could be obtained from the crystal structure of CsA if, in addition to allowing the MeBmt side chain to rotate, the backbone structure of CsA was allowed to move during a conformational search. Normally, this is accomplished in cyclic systems by defining a ring-closure bond[23] and allowing backbone torsional angles to rotate. In view of the complexity of the CsA molecule, which has 33 ring torsional angles, a conformational search of this magnitude would present two major problems. Clearly, massive computer resources would be required. Yet even if this problem could be circumvented via the use of a supercomputer, one would still be faced with the formidable task of analyzing the large volume of data generated (i.e., hundreds of thousands of conformations).

Several shortcuts have traditionally been used to reduce the number of conformers resulting from a conformational search. One technique typically employed involves the use of an energy cutoff during the search procedure whereby all conformations with energy values a specified amount above the minimum energy conformation are eliminated. However, this practice may be risky. It has been reported that some of the high-energy structures generated during a conformational search of a cyclic pentapeptide actually ended up being the lowest energy conformations after minimization.[29] Had energy been used as a constraint in that study, some of the most stable structures would have been rejected. In any case, the 400 conformers we obtained from the conformational search of the crystal structure of CsA (in which four torsional angles were systemically varied by 10° increments) spanned an energy range of only about 7 kcal/mol. Therefore, in this example it is readily apparent that the use of an energy constraint would not be a viable means of curtailing the number of conformers requiring subsequent analysis.

Another shortcut commonly utilized to decrease the number of conformers generated during a search is to increase the angle increment employed. We stress that this practice should be approached with caution. Using an angle increment that is too large may result in overlooking significant numbers of viable conformers or, in fact, entire families of conformations. Again referring to the results obtained for the crystal structure of CsA, the use of 30° angle increments in a conformational search employing four torsional angles gave zero viable conformations. Furthermore, a 20° angle increment for χ_1 and 30° angle increments for χ_2, χ_3, and χ_4 neglected significant regions of angle space that the MeBmt side chain can occupy (section VI.A). These regions clearly show up when 10° angle increments are used. On the other hand, the use of 3° angle increments did not produce any additional clusters (or families) of conformers than those resulting from the 10° incremental search. These data clearly show that employing an angle increment larger than 10° would not be a satisfactory method for reducing the number of CsA conformers and illustrate how easy it can be to overlook viable conformations when too large angle increments are used. It was obvious to us that 30° increments were too large (zero conformations were obtained), but it would not have been obvious that the combined 20° and 30° increment results missed viable conformations without our having carried out the more exhaustive 10° and 3° incremental searches. These results suggest that viable conformations could be missed when searches are carried out on other organic molecules using angle increments of 30°, 60°, or even 120° increments, especially in those cases in which the organic molecule contains unusual structural features.

With these limitations defined, we decided to carry out a limited conformational search of the 1-position residue only (i.e., four torsional angles) for both the crystal structure and the apolar solution structure of each analogue. The use of two structures of each molecule enabled us to generate both the extended and the folded conformations of the MeBmt analogues without the need to define a large number of torsional angles or a ring-closure bond. Since fewer torsional angles needed to be varied, the complexity of each computation was kept minimal. On average, each computation required less than 1 min of CPU time.

Data Analysis. The first stage of data analysis typically involves refining the structures of conformers via energy minimization and eliminating any duplicate structures. The conventional approach toward structure refinement is to minimize each viable conformer generated during a search, but this phase of analysis can be extremely time-consuming and may not be practicable without the use of a supercomputer. After all conformers are energy minimized, duplicates are eliminated by examining the energy and geometry of each conformer and comparing them to those of every other conformer. If a large number of structures has been minimized, this procedure can quickly reach impracticable dimensions. Understandably, one of the principle issues regarding the analysis of conformational search results concerns data reduction. Several means of reducing the number of conformers generated during a conformational search were described above. In this section we describe a way to reduce the number of conformers generated after a conformational search has completed but prior to energy minimization and compare this with other

(28) Howard, A. E.; Kollman, P. A. *J. Med. Chem.* **1988**, *31*, 1669.

(29) Richards, A.; Simeroth, P., unpublished data obtained by using RINGSEARCH on cyclo(Ala)$_5$.

methods commonly employed. Hence, rather than altering the setup of the search, we consider various techniques of sampling its results (i.e., conformation filtering).

For example, one could employ a random means of sampling the search results.[30] In this approach, a specified number of conformers would be randomly discarded, leaving the balance to undergo analysis; the conformers retained would be taken to represent the system under consideration. This approach might be feasible if the energy, intramolecular interactions, etc., were the same for each conformer. However, one can see that, for CsA, such a casual procedure would not provide an effective means of reducing the data to be analyzed. In fact, random sampling would likely lead to the rejection of critical sets of conformers and should thus be considered a risky practice.

Alternatively, one might elect a more selective approach toward sifting through large numbers of conformers. Typically, the notion of grouping conformers into "families" is appealing. So if one could accurately identify all the conformational families of a given molecule, then the various families could be sampled by selecting representative members from each.[30] The difficulty with this technique lies in the definition of a conformational family. In general, a conformational family is considered to consist of a group of conformers that, when superimposed, occupy a relatively small region of three-dimensional space and that typically converge to the same local minimum upon energy minimization. These families can often be visualized in angle maps as compact clusters of dots. However, it is often difficult to determine the boundaries of a conformational family or the precise neighborhood in which it resides.[30] This is especially apparent in instances where many dots (conformers) are spread over a large, diffuse region of angle space. In such cases, the neighborhoods of two or more conformational families overlap, making it impossible to discern their respective boundaries. Therefore, since a continuous range of dots may contain members from several different conformational families, it would not be possible to accurately select representative members from each distinct conformational family.

In this study, we found that nonrandom sampling of search results resolved the problem of data reduction more satisfactorily than the methods described above. The exact procedure that we employed, described in detail in section VI.A, was designed to systematically eliminate any conformer if all four of its torsional angles χ_1–χ_4 were within 30° of each of the corresponding torsional angles of a conformer already selected for energy minimization. This objective was accmplished in a two-step process. In the first step, a conformational search of each structure was carried out using as large an angle increment as possible (up to a maximum of 30°) for each of the torsional angles. When zero conformers resulted from a particular search, then the angle increment of one or more of the torsional angles was reduced. This approach provided a course filter for weeding out undesired conformers and enabled us to quickly examine vast regions of angle space. However, it has been demonstrated in detail (vide supra) that if too large an angle increment is used, a significant number of conformers will be missed. Therefore, during the second step of this procedure we were required to examine the neglected regions of angle space more closely. It was necessary to determine precisely which conformers had been overlooked. This was accomplished by comparing the results of different searches as a function of dihedral angle increment. Conformers from the more exhaustive searches were then used to repopulate angle space neglected in the less rigorous searches. While this method of data reduction may appear tedious, in actuality it required minimal time and effort.

B. Influence of Methyl Configuration and Degree of Substitution of MeBmt in Determining Stable Conformations of CsA. The cyclosporin system is one of the most difficult peptides to alter without drastically reducing biological activity. As noted previously, deletion of carbon atoms from such apparently widely separated locations on the cyclic undecapeptide ring system as the MeBmt[1] and MeLeu[6] positions, leads to dramatic losses in immunosuppressive activity.[9,11] Numerous additional examples of seemingly subtle modifications resulting in significant reductions in biological activity have been reported by the Sandoz group.[8]

This study was initiated with the objective of identifying how the conformational preferences of the 1-position side chain of CsA are altered as the C4 carbon is modified by addition or deletion of methyl groups. The results we have described establish that the (4*R*)-methyl group in MeBmt of CsA stabilizes conformations that fall within the XTL family, that is, conformations in which the 1-position side chain is folded across the cyclic undecapeptide ring system. Deletion of the C4 methyl group, as in (MeBth)[1]CsA, or epimerization at C4, as in ((4*S*)-MeBmt)[1]CsA, allows the 1-position side chain to adopt additional conformations in which it rotates away from the peptide ring system. The Boltzmann distributions for these two analogues suggest that their 1-position residues can access XTL conformers but only with much less frequency than do CsA and ($MeBm_2t$)[1]CsA. These results show the importance of the configuration of a single methyl group in a cyclic peptide system of 1202 molecular weight.

Our results also show that the addition of a (4*S*)-methyl group to MeBmt, as in ($MeBm_2t$)[1]CsA, restricts the conformations accessible to the 1-position side chain. Addition of methyl groups to the α-carbon of amino acids is known to restrict the conformational space available to peptides containing an α-methylated amino acid[32] and has been used to define possible conformations for biologically active peptides. The addition of the extra methyl group at C4 in MeBmt serves a similar function as indicated by the reduced number of conformations and more restricted angle space available to ($MeBm_2t$)[1]CsA. We use this property of $MeBm_2t$ in the following section to try to identify the bioactive conformation of CsA.

C. Proposed Bioactive Conformation of CsA Analogues. In order to understand the drug–receptor interactions for CsA, we would like to identify the bioactive conformation of CsA, i.e., the conformation of CsA when it is bound to the receptor. Unfortunately, the identity of the receptor with which CsA interacts remains largely unresolved.[33-35] All four of the CsA analogues considered here have some degree of immunosuppressive activity (see Table I), and if we assume that they all interact with the same receptor molecule via the same mode of action, then it seems reasonable that they are all able to adopt a common conformation that corresponds to the bioactive conformation. To determine if the four CsA analogues can adopt a common conformation, it was necessary to identify all possible conformations of each molecule. The conformations of the 1-position residue of CsA and three CsA analogues determined in vacuo through a series of conformational searches are reported in Tables V–VIII. In the discussion that follows, we will examine more closely the conformations that the four CsA analogues have in common and then, on the basis of the population analysis and its effect on the observed dissociation constant, determine whether it is possible to correlate the biological activity of a given molecule with the equilibrium constant for formation of the common conformation(s).

Our results show that the bioactive conformation is not found in the XDV family. Because the two cyclosporin analogues with relatively low immunosuppressive activity, ((4*S*)-MeBmt)[1]CsA and (MeBth)[1]CsA, can adopt conformations derived from the XDV family of conformers, but CsA and ($MeBm_2t$)[1]CsA do not, the XDV conformers cannot represent the bioactive conformation of the cyclosporins. Furthermore, since the conformers belonging to the XDV conformational family are generally lower in energy than those that are members of the XTL (or SOL) conformational

(30) For a discussion of various methods of sampling the results of a conformational search, see: *TRIPOS TECHNICAL NEWSLETTER*; Tripos Associates: St. Louis, MO, Jan 1988.
(31) Miller, K. E., unpublished data.
(32) Marshall, G. R.; Gorin, F. A.; Moore, M. L. *Annu. Rep. Med. Chem.* **1978**, *13*, 227.
(33) Handschumacher, R. E.; Harding, M. W.; Rice, J.; Drugge, R. J.; Speicher, D. W. *Science* **1984**, *226*, 544.
(34) Colombani, P. M.; Robb, A.; Hess, A. D. *Science* **1985**, *228*, 337.
(35) LeGrue, S. J.; Turner, R.; Weisbrodt, N.; Dedman, J. R. *Science* **1986**, *234*, 68.

families, the calculations predict that more ((4S)-MeBmt)^{1}CsA and (MeBth)^{1}CsA molecules will be in the nonactive XDV conformations than in the biologically active (XTL or SOL) conformation(s). This reasoning is consistent with the observed lower immunosuppressive activity of ((4S)-MeBmt)^{1}CsA and (MeBth)^{1}CsA.

Our results also indicate that the deuterochloroform solution structures for CsA and its analogues are not important contributors to the equilibrium conformations. Recently, van Gunsteren and co-workers have reported that molecular dynamics calculations indicate that the hydrogen bond between the (3R)-hydroxyl group and the MeBmt carbonyl oxygen that stabilizes the deuterochloroform CsA conformation is replaced in the polar CsA conformation by hydrogen bonds to water. Moreover, their results show that the CsA conformation in water is remarkably similar to the X-ray conformation.[27] Experimental evidence for the folded conformation in aqueous media also has been reported. Quesniaux et al. found that the binding of 1-position analogues of cyclosporin to monoclonal antibodies was consistent with a folded (X-ray) conformation.[36] Thus, our molecular mechanics calculations, which do not include solvent, are in accord with both the molecular dynamics simulations and the results of antibody binding in aqueous media. Since this agreement could be purely fortuitous, it will be important to conduct future computations on CsA in a solvent environment in order to compare the in vacuo energies determined here with those computed in solution. The results of such a study will help to determine the magnitude of in vacuo energetic errors in the present study and their impact on the conclusions drawn herein.

We now examine if the relative stability of the XTL conformations of each CsA analogue correlates with the corresponding immunosuppressive activity. There are two XTL conformations that all four molecules have in common (see section VIII.E). Based on the torsional angle values of the MeBmt analogues, the data indicate that (MeBm$_2$t)^{1}CsA conformer A corresponds most closely to ((4S)-MeBmt)^{1}CsA conformer D, (MeBth)^{1}CsA conformer K, and CsA conformer D; whereas, (MeBm$_2$t)^{1}CsA conformer B is most similar to conformer F of ((4S)-MeBmt)^{1}CsA, conformer B of (MeBth)^{1}CsA, and conformers A and B of CsA. Because (MeBm$_2$t)^{1}CsA has less immunosuppressive activity than CsA, (MeBm$_2$t)^{1}CsA should spend less time in the bioactive conformation if its reduced immunosuppressive activity is caused only by changes in conformational populations and not by other factors, such as steric hindrance with the receptor. Thus, conformer B of the MeBm$_2$t analogue appears to represent the conformation that acts at the receptor.[37]

The Boltzmann distributions we have calculated for the four CsA analogues are consistent with the relative immunosuppressive activities of these compounds. According to our calculations, 64% of the cyclosporin molecules will occupy conformers A and B, which most closely resemble conformer B of (MeBm$_2$t)^{1}CsA. If these conformers represent the bioactive conformation of CsA, then their combined Boltzmann population must be compared to the immunosuppressive activity of CsA presented in Table I. The fraction of molecules adopting the bioactive conformation(s) of CsA (i.e., the sum of the Boltzmann populations of CsA conformers A and B) represents the concentration of biologically active conformers of CsA, $[C^A]_{CsA}$, relative to that of the inactive CsA conformers. The analogue conformers that are closest in structure to conformers A and B of CsA are (MeBm$_2$t)^{1}CsA conformer B, [(4S)-MeBmt]^{1}CsA conforme F, and (MeBth)^{1}CsA conformer B. If we assume that K_d^{int} for each analogue is the same as for CsA (i.e., the C4 modifications do not interfere in the binding to the receptor), then the relative immunosuppressive activity of each CsA analogue can be calculated from the Boltzmann population of the bioactive conformers according to eq 5 as shown in Table IX.[38]

Table IX. Immunosuppressive Activity and Boltzmann Population of Geometrically Similar Conformers of CsA, (MeBm$_2$t)^{1}CsA, ((4S)-MeBmt)^{1}CsA, and (MeBth)^{1}CsA

compd	conformer[a]	Boltzmann population,[b] %	predicted IS activity rel to CsA,[c] %	obsd IS activity,[d] %
CsA	A and B	64	100	100
(MeBm$_2$t)^{1}CsA	B	22	34	20–30
((4S)-MeBmt)^{1}CsA	F	8	12	2–4
(MeBth)^{1}CsA	B	24	37	10–13

[a] Conformers are defined in Tables V–VIII. [b] Relative populations determined from a Boltzmann distribution and reported in Tables V–VIII. [c] Predicted relative immunosuppressive activity (IS) calculated from $[([C^A]_{analogue}/[C^A]_{CsA}) \times 100]$, for case where K_d^{int} is the same for all analogues, using the values in Tables V–VIII. [d] See Table I.

As one can see from Table IX, the computational results are in fairly good agreement with the experimental data in that the calculated values predict that the synthetic CsA analogues will all be less active than CsA. The correlation between the biological activity of (MeBm$_2$t)^{1}CsA and CsA and the Boltzmann distributions of their respective side chains is remarkably good and deserves further comment. As described in preceding sections, the added (4S)-methyl group in (MeBm$_2$t)^{1}CsA restricts the conformations accessible to the MeBm$_2$t side chain. The angle maps (Figure 3b) and the Boltzmann distributions (Table IX) show that the MeBm$_2$t analogue can access the proposed bioactive conformation approximately one-third as frequently as can CsA. Thus, on the basis of a comparison of the side-chain populations only, (MeBm$_2$t)^{1}CsA would have about 34% the immunosuppressive activity of CsA. In fact, the immunosuppressive activity of (MeBm$_2$t)^{1}CsA lies between 20 and 30% that of CsA when measured in the thymocyte assay (Table I), is 25% for inhibition of IL-2 release,[39] and averages 35% when measured in lymphocytes.[39] This truly remarkable agreement between side-chain conformational population and biological activity requires that either the added methyl group functions only to restrict side-chain conformations (i.e., it does not interact favorably or unfavorably with the immunosuppressive receptor) or it gives rise to two (or more) factors that cancel each other, such as enhanced bioactivity due to a constrained conformation offset by an unfavorable steric interaction between the added C4 methyl group and the CsA receptor.

It is reasonable that the correlation between the Boltzmann distributions of the 1-position side chains in CsA and (MeBm$_2$t)^{1}CsA that we have determined in vacuo would carry over into aqueous media because of the close similarity between CsA conformers A and B and (MeBm$_2$t)^{1}CsA conformer B and the fact that molecular dynamics calculations[27] determined that the conformations of CsA in vacuo and water are very similar. The relative stabilities of these two analogues are probably primarily a reflection of small destabilizations of (MeBm$_2$t)^{1}CsA conformer B by the added methyl group. However, the nature of the XDV conformations calculated in vacuo are such that we anticipate they will not be retained in aqueous media to the same extent as the XTL conformations. This is because the side chain in the XDV conformers projects away from the hydrophobic

(36) Quesniaux, V. F. J.; Wenger, R. M.; Scmitter, D.; van Reganmortel, M. H. V. *Int. J. Peptl. Protein Res.* **1988**, *31*, 173.

(37) (MeBm$_2$t)^{1}CsA spends 77% of its time in conformation A, which is a larger percentage of time than CsA spends in any given conformation or in A and B combined.

(38) Interestingly, the Boltzmann population of the proposed bioactive conformation of CsA is only 64%. This suggests that it may be possible to design a conformationally constrained analogue of CsA for which the Boltzmann population of the bioactive conformation is greater than 64%, and therefore the corresponding immunosuppressive activity is greater than 100% that of CsA.

(39) Additional biological data: suppression of IL-2 secretion, 25%; inhibition of ionomycin and PMA stimulation, 27%, 35%, 37%, 41% (four assays). Data kindly provided by Drs. J. Boger and P. Durette, Merck Sharp & Dohme.

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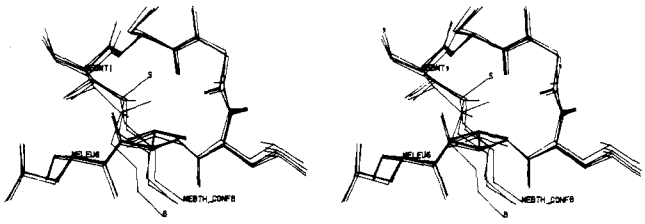

Figure 4. Relaxed stereo representation of residues 1–6 of the proposed bioactive conformation of CsA and three CsA analogues. Conformers B of $(MeBm_2t)^1$CsA and $(MeBth)^1$CsA are labeled B and MEBTH_CONFB, respectively, while conformer F of [(4*S*)-MeBmt]^{1}CsA is labeled with an S. The unlabeled conformer is CsA conformer B.

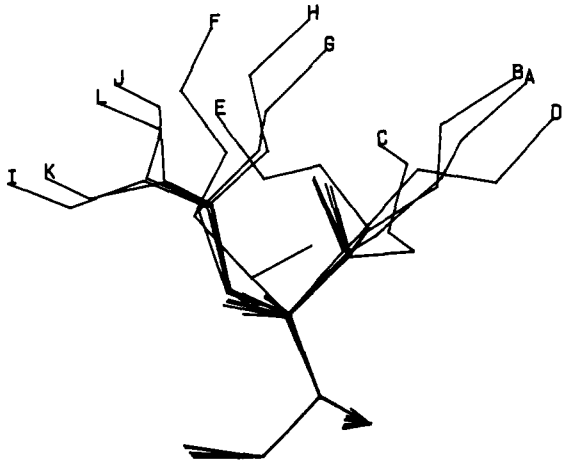

Figure 5. Superposition of the 12 unique conformations of the MeBmt side chain in CsA resulting from conformational searches and subsequent energy minimizations.

cyclosporin peptide backbone into a vacuum for the molecular mechanics calculations but into an aqueous (or partially aqueous) environment in the in vitro assay medium. New hydrophobic interactions are likely to form in the aqueous environment that would alter the relative stabilities of the XDV conformers. For these reasons, the quantitative relationship between the Boltzmann distribution calculated in vacuo and immunosuppressive activity for the ((4*S*)-MeBmt)^{1}CsA and $(MeBth)^1$CsA conformers is likely to change in aqueous media. It should be possible to calculate the relative stabilities of the water-solvated conformers, but this would require about 10 h of CPU time on a supercomputer for each conformer[27] and is clearly beyond the present study. The conformations listed in Tables V–VIII will provide good starting conformations for simulations of the conformations in aqueous media.

The proposed bioactive conformation is compatible with the results of Quesniaux et al. who concluded on the basis of antibody binding studies that CsA adopts a conformation in aqueous media in which the MeBmt side chain is folded across the cyclic undecapeptide ring system.[36] The average side-chain orientations of the 1-position residue of the average of conformers A and B of CsA, conformer B of $(MeBm_2t)^1$CsA, conformer F of ((4*S*)-MeBmt)^{1}CsA, and conformer B of $(MeBth)^1$CsA are $\chi_1 = 191°$, $\chi_2 = 64°$, $\chi_3 = 177°$, and $\chi_4 = 225°$. A relaxed stereo representation of the proposed bioactive conformations of CsA and three analogues analyzed in this paper is presented in Figure 4, and corresponding coordinate files are provided in the supplementary material (ordering information is provided on the masthead page).

One of the interesting consequences of the proposed bioactive conformation of CsA is that the side chains of two critical residues for immunosuppressive activity, MeLeu^6 and MeBmt^1, are adjacent to each other in spite of being positioned transannularly in the CsA 33-membered ring system. As noted previously, removal of three carbons from either of these side chains leads to dramatic losses in immunosuppressive activity.[9,11] The proposed

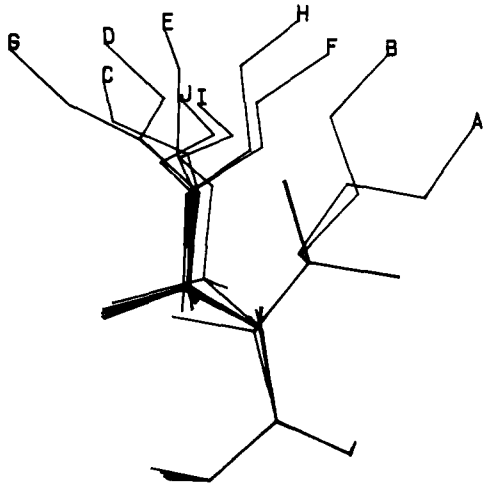

Figure 6. Superposition of the 10 unique conformations of the $MeBm_2t$ side chain in $(MeBm_2t)^1$CsA resulting from conformational searches and subsequent energy minimizations.

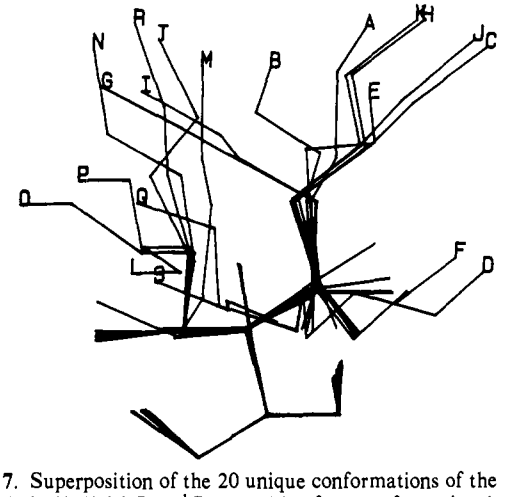

Figure 7. Superposition of the 20 unique conformations of the MeBmt side chain in ((4*S*)-MeBmt)^{1}CsA resulting from conformational searches and subsequent energy minimizations.

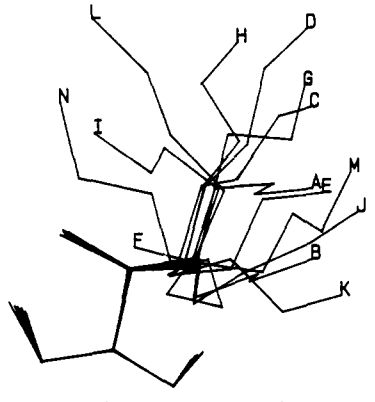

Figure 8. Superposition of the 14 unique conformations of the MeBth side chain in $(MeBth)^1$CsA resulting from a conformational search of the model derived from the crystal structure of CsA followed by energy minimizations.

bioactive conformation places these 3-carbon fragments very close to each other so that either they bind simultaneously to the CsA receptor or they interact with one another. We have carried out preliminary calculations to evaluate this latter possibility (data not shown). The results of these calculations suggest that the MeBmt side-chain conformational distribution in $(MeAla)^6$CsA does not greatly differ from that described here for MeBmt in CsA nor does the MeLeu^6 side-chain conformational distribution in $(MeLeu(3\text{-}OH))^1$CsA differ from that of MeLeu^6 in CsA.[31]

Therefore, we presently favor a direct interaction between the isobutyl side chain in MeLeu[6] with the receptor to explain the loss of biological activity reported for (MeAla)[6]CsA. Similarly, an independent interaction between the butenyl side chain in MeBmt with the receptor may account for the dramatically reduced immunosuppressive activity of (MeLeu(3-OH))[1]CsA.

The use of the conformational search and energy minimization strategy we have described above has provided insight into the effects of the primary structure of this peptide on its conformations and biological activity. The proposed bioactive conformation serves as a standard against which to evaluate the potential biological activity of new CsA analogues. Further studies to compare the conformational preferences of 1-position CsA analogues with the immunosuppressive activities are in progress.

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Registry No. CsA, 59865-13-3; $(MeBm_2t)^1$CsA, 114865-22-4; ((4*S*)-MeBmt)[1]CsA, 122090-69-1; (MeBth)[1]CsA, 114891-20-2; MeBmt, 59865-23-5.

Supplementary Material Available: Tables of energetic components of the total strain energy of CsA, $(MeBm_2t)^1$CsA, ((4*S*)-MeBmt)[1]CsA, and (MeBth)[1]CsA, figures of the superposition of unique conformations of those given above, and listings of coordinate files of MeBmt (conformers A and B), MeBth (conformer B), $MeBm_2t$ (conformer B), and (4*S*)-MeBmt (conformer F) (34 pages). Ordering information is given on any current masthead page.

Surface-Enhanced Raman Spectroscopic Investigation of Human Immunoglobulin G Adsorbed on a Silver Electrode

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Abstract: SERS spectra of human IgG adsorbed on a silver electrode from solution concentrations of 1.0 μM IgG in pH 7.2 phosphate buffer are presented and spectral assignments made. Laser power of 50 mW was sufficient to obtain high-quality spectra. Overall enhancement was in the range of 10^4–10^5. The spectrum consisted only of vibrations from amino acid residues with functional groups that would complex with silver, including tyrosine, tryptophan, cystine, and acidic groups. No amide backbone stretches were observed. From the relative intensities of the vibrations, average conformations of the adsorbed amino acid groups were proposed. The selective enhancement, afforded to adsorbed residues, allowed monitoring of protein conformational changes at low coverage in high buffer ionic strength.

Recently, several groups have used surface-enhanced Raman spectroscopy (SERS) and surface-enhanced resonance Raman spectroscopy (SERRS) to characterize biological molecules. Koglin and Sēquaris conducted in-depth investigations of nucleic acids and DNA with silver colloids ref 1 and references therein. Several amino acids have been also studied by using silver colloids to obtain SERS.[2-6] The aromatic amino acids, tyrosine (Tyr), phenylalanine (Phe), tryptophan (Trp), and histidine (His), exhibited intense spectra[2-4] but displayed great variations in relative peak intensities and frequencies from study to study. Methods of preparation and age of the colloid, as well as amino acid concentration, greatly affected the SERS spectra. Some of these experiments used the spectrum to predict the orientation of the amino acids on the colloidal silver.[2,5,6] The researchers discovered that all of the amino acids bound to the silver through their carboxyl groups except for cystine, which was attached through a strong silver–sulfur bond.[6] It would be expected that SERS spectra for proteins would differ considerably from those of amino acids since the carboxyl groups would be incorporated in the amide bonds of the skeletal structure of the protein and become inaccessible for binding to the silver. Cotton and Van Duyne examined metal-containing biomolecules including myoglobin, cytochrome *c*,[7] and chlorophyll pigments[8] adsorbed on silver electrodes using SERRS. This technique yielded high-quality spectra, but the resonance enhancement only probed the region of the molecule surrounding the metal center. Again, nonresonance spectra would be expected to emphasize very different features.

A few attempts have been made to generate SERS spectra of amino acids[9] and the proteins bovine serum albumin and lysozyme[10] at electrodes, and the resulting spectra were rather weak and featureless. The SERS electrode spectra of leucine–isoleucine–valine binding and leucine-specific proteins have also been observed.[11] The goal of the present work was extension of SERS to the study of large proteins. In this paper, the spectrum of human immunoglobulin G (IgG) is reported and vibrational assignments made. Based on these results, adsorptive behavior is described as a function of electrode potential and ionic strength.

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