Supplementary Material Available: Experimental procedures, including analytical and spectial data, for the preparations of 7 and 14 (7 pages). Ordering information is given on any current masthead page.

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NMR Studies of an FK-506 Analogue, [U-^{13}C]Ascomycin, Bound to FKBP: Conformation and Regions of Ascomycin Involved in Binding

FK-506 is a potent immunosuppressant[1,2] that blocks T cell activation by inhibiting the production of lymphokines at the transcription level. Although its precise mechanism of action is unknown, its immunosuppressive activity appears to be related to the binding of FK-506 to a 108 amino acid protein from human T cells.[3,4] This FK-506 binding protein (FKBP) has been shown to be a peptidyl-prolyl cis-trans isomerase that is inhibited by FK-506[5,6] and the structurally related immunosuppressant, rapamycin.[6]

The X-ray crystal structure of FK-506 has been determined in the absence of FKBP[7] and used to aid in the design of FK-506 analogues.[8] In addition, the three-dimensional structure of FK-506 in chloroform solution has been recently obtained by NMR spectroscopy.[9] However,

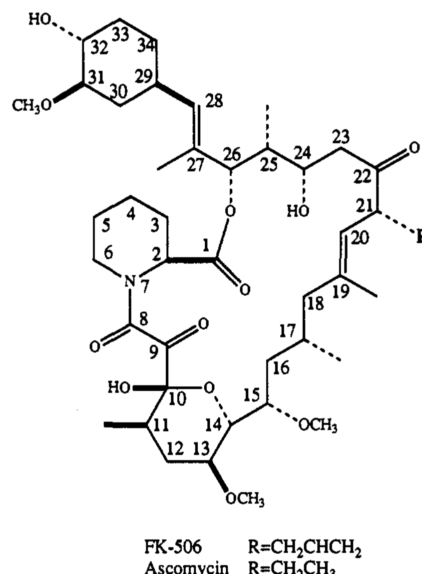

Figure 1. Structures of FK-506 and ascomycin.

as recently demonstrated by NMR studies of cyclosporin A bound to cyclophilin,[10-12] the conformation of a molecule when bound to its target site may be very different from its uncomplexed conformation determined in solution or in the crystalline state. In order to aid in the design of FK-506 analogues that are clinically useful as immunosuppressants, it would be of value to determine the conformation of FK-506 when bound to FKBP and to identify those portions of FK-506 that interact with the protein. To date, the ^{13}C carbonyl chemical shifts of C8 and C9[13] and the ^{1}H chemical shifts of the piperidine ring of FK-506 when bound to FKBP have been reported.[14] From the upfield shifts of the piperidine ring protons of FK-506 and rapamycin and NOEs observed between these protons and aromatic protons of FKBP, tentatively assigned to Trp59, Tyr89, and Phe99, it was concluded that the common pipecolinyl moiety of FK-506 and rapamycin is involved in binding to FKBP.[14]

In this report we describe NMR studies of a uniformly ^{13}C-labeled FK-506 analogue, ascomycin,[15] bound to recombinant human FKBP. Ascomycin, also known as FR-

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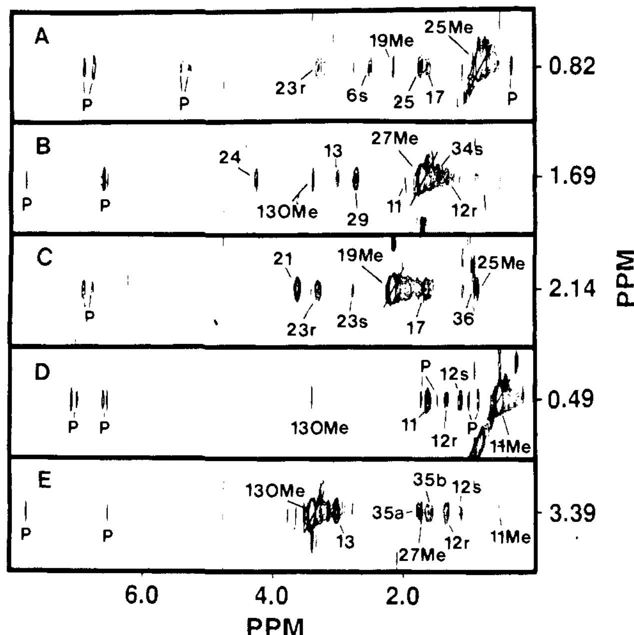

Figure 2. (A-E) Cross sections (ω_2, vertical axis; ω_3 horizontal axis) from a 3D ^{13}C HMQC-NOESY spectrum[24] of the [U-^{13}C]-ascomycin/FKBP complex acquired with a mixing time of 40 ms at the ^{13}C chemical shifts (ω_1): (A) 11.3, (B) 11.3, (C) 19.2, (D) 16.5, and (E) 56.8 ppm. The diagonal peaks are indicated in each of the planes by a solid line. The 3D data set was collected at 30 °C, on a Bruker AMX600 spectrometer as a series of 40 complex (t_1) 2D experiments [64(t_2) × 2048(t_3)] with use of a spectral width of 6493.5 Hz in ω_1 and 8620.7 Hz in ω_2 and ω_3. Thirty two scans were collected per t_3 experiment, and the spectrum was processed on a Silicon Graphics 4D/220GTX computer by using in-house-written software. The [U-^{13}C]ascomycin was produced by growing *Streptomyces hygroscopicus* subsp. *ascomyceticus* UV125, a strain derived from ATCC 14891, in a chemically defined fermentation medium with [U-^{13}C]glucose(99%) as the sole carbon source. The [U-^{13}C]ascomycin, that was ^{13}C-labeled to a level of greater than 95%, was extracted from the broth with XAD-2 polystyrene resin, the methanol eluate from which was triturated with ethyl acetate. The purification of [U-^{13}C]ascomycin was achieved by countercurrent chromatography in a solvent system of hexane/ethyl acetate/methanol/water (70:30:15:6). Recombinant human FKBP was cloned from a Jurkat T cell cDNA library and expressed in *Escherichia coli* by using the pKK233-2 vector containing a *trc* promoter. FKBP was isolated from these cells by using ion-exchange and size-exclusion chromatography. Both the cloning and purification will be described in detail elsewhere. The NMR sample was ~3 mM [U-^{13}C]ascomycin/FKBP in a ^2H_2O solution containing sodium phosphate (50 mM), sodium chloride (100 mM), and [$^2H_{10}$]dithiothreitol (5 mM), pH = 6.5.

900520,[16] only differs from FK-506 by having an ethyl versus an allyl side chain at C21 (structures shown in Figure 1). Like FK-506, ascomycin is a potent immunosuppressant[16] that binds with approximately the same affinity to FKBP as FK-506.[17] Here, we report on the conformation of ascomycin when bound to FKBP and those portions of ascomycin that interact with the protein on the basis of NOEs observed between ascomycin and FKBP.

Assignments. The NMR signals were assigned from an analysis of two-dimensional $^1H/^{13}C$ and $^{13}C/^{13}C$ correlation spectra and NOE data. From the pattern of one-bond carbon–carbon correlations observed in a ^{13}C COSY spectrum of [U-^{13}C]ascomycin bound to FKBP, carbons 1–6, 8–36, and side chain methyl carbons (11-Me, 17-Me, 19-Me, 25-Me, 27-Me) were easily identified. The protons attached to these carbons were assigned from a heteronuclear multiple quantum correlation (HMQC) experiment.[18] The protons of the three methoxy groups were distinguished from one another upon examination of the NOE data, and their attached carbons were identified from the HMQC experiment.

Bound Conformation of Ascomycin. The conformation of ascomycin when bound to FKBP was determined from NOEs observed in a 3D HMQC-NOESY spectrum of the [U-^{13}C]ascomycin/FKBP complex (Figure 2). By using the two-spin approximation, 88 proton–proton distances were calculated from the integrated 3D NOE cross-peak volumes measured from the 3D NOE data set acquired with a mixing time of 40 ms (Figure 2). These distance constraints were used to generate 200 initial structures with the DSPACE distance geometry algorithm (Hare Research). These initial structures were subsequently refined by using a dynamical simulated annealing protocol with the XPLOR program.[19] Figure 3 shows a superposition of the 20 lowest energy structures. The structure of ascomycin when bound to FKBP was well defined by the NOE data as evidenced by the small average rms deviation (0.45 ± 0.17 Å) of the heavy atoms to a calculated average structure. The conformation of the macrocyclic ring is defined by several key NOEs. The NOE between 25-Me and H6s[20] (Figure 2A) defines the

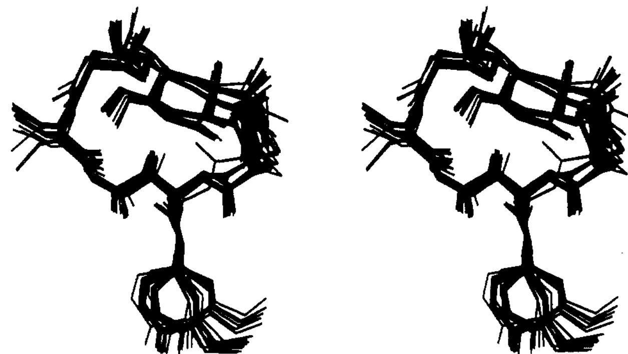

Figure 3. Stereoview of the superposition of 20 structures generated by distance geometry simulated annealing calculations from 88 NOE-derived proton–proton distance constraints by using DSPACE and XPLOR programs, respectively. The simulated annealing protocol that was employed[11] consisted of 5 ps of restrained molecular dynamics at 1000 K followed by slow cooling from 1000 to 300 K in steps of 10 K. The final stage consisted of 600 steps of restrained energy minimization. From 200 starting structures generated by using DSPACE that were further refined with XPLOR, 73 structures containing a trans amide bond were selected on the basis of the lowest empirical energy and NOE violations. None of these structures violated the NOE constraints greater than 0.2 Å. The superimposition of the heavy atoms of these structures on the calculated average gives an average RMSD of 0.45 ± 0.17 Å.

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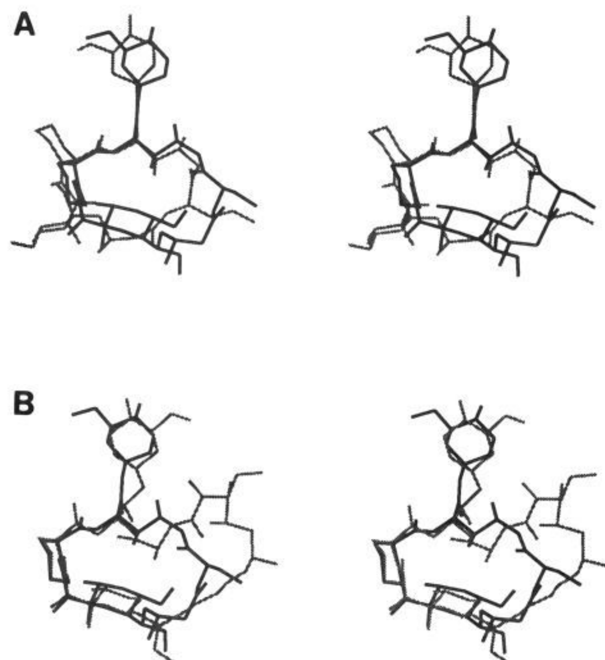

Figure 4. Stereoviews of the superposition on the common heavy atoms of the average NMR structure of bound ascomycin (bold) over the X-ray structures of uncomplexed FK-506 (A) and rapamycin (B).

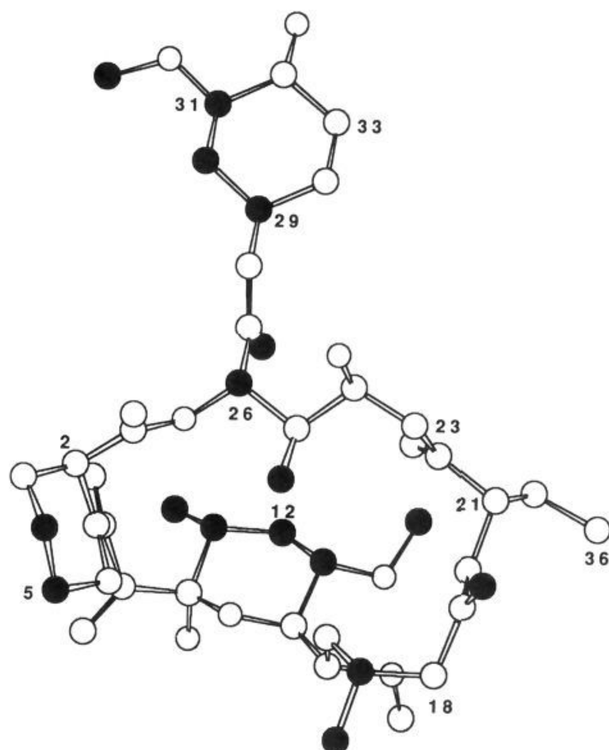

Figure 5. Three-dimensional structure of ascomycin bound to FKBP. Protons on the ligand which showed NOEs to the protein are denoted by a black shading of the carbons to which they are attached. Although no NOEs were observed from the protons at position 3 to the protein, the upfield shift of their resonances, −1.09 and 0.25 ppm, suggests that they are in close proximity to an aromatic region of FKBP.

orientation of the piperidine ring. The 27-Me group is on the opposite side of the macrocyclic ring to 25-Me in a location close to the pyranose ring as evidenced by the NOEs from 27-Me to H12*r*, H13 and 13-OMe (Figure 2, parts B and E). The remaining portion of the macrocyclic ring (region 19–23) is defined by NOEs from 19-Me to H17, H21, H23*r*, H23*s*, and 25-Me (Figure 2C) and NOEs from 13-OMe to both methylene protons (H35) of the ethyl side chain (Figure 2E). The orientation of the cyclohexyl side chain relative to the macrocyclic ring is characterized by NOEs between the 27-Me group and H29 and H34*s* (Figure 2B).

Although there are no protons in the tricarbonyl (position 8–10) region of ascomycin, the numerous NOEs observed for the remainder of the molecule were able to help define this portion of the structure. Our structure calculations utilized a 2-fold dihedral angle term (CHARMM)[21] to allow transitions between the cis and trans conformations of the amide bond. On the basis of these calculations, the lowest energy structures that met the NMR constraints mostly contained a trans amide bond. Within 10 kcal/mol of the lowest energy structure, only two structures were obtained with a cis amide bond; whereas, 33 contained a trans amide. Thus, our results suggest that ascomycin adopts a trans amide bond when bound to FKBP.

Figure 4 depicts the average NMR structure of ascomycin (bold) bound to FKBP superimposed on the three-dimensional structures of FK-506[7] (Figure 4A) and rapamycin[22] (Figure 4B) determined in the absence of FKBP by X-ray crystallography. As shown in Figure 4A, the conformation of ascomycin is very different from uncomplexed FK-506. Unlike ascomycin when bound to FKBP, the conformation of FK-506 in the free state is characterized by a cis amide bond and a different orientation of the piperidine and pyranose rings. The three-dimensional structure of bound ascomycin determined by NMR spectroscopy more closely resembles the X-ray structure of uncomplexed rapamycin. As shown in Figure 4B, the common atoms of both molecules superimpose very well, except for the opposite orientation of the cyclohexyl ring. In the crystalline state, rapamycin adopts a trans amide bond at the 9,10 position which probably contributes to the resemblance of these structures.

Portions of Ascomycin in Close Proximity to FKBP. In Figure 5, those portions of ascomycin that interact with FKBP are schematically illustrated. The protons attached to the filled carbon atoms exhibit NOEs to FKBP in 3D HMQC-NOESY spectra. In agreement with previously described NMR studies of the FK-506/FKBP complex,[14] NOEs were observed between the piperidine ring of ascomycin and aromatic protons of FKBP. These NOE data and the upfield chemical shifts of H3*r*,*s* (0.25, −1.09 ppm), H4*r*,*s* (−0.38, −1.87 ppm), and H5*r*,*s* (−1.01, −0.71 ppm) clearly indicate that the piperidine ring of ascomycin interacts with aromatic amino acids of FKBP. A portion of the pyranose ring must also be in close proximity to FKBP as evidenced by NOEs from 11-Me (Figure 2D), H11, H12, H13, and 13-OMe (Figure 2E) to the protein. NOEs were also observed between some of the cyclohexyl side chain protons (H29, H30, H31, and 31-OMe) and FKBP, indicating that part of the cyclohexyl ring of ascomycin is also involved in binding to the protein. These results are consistent with the dramatic loss of binding of an FK-506 analogue that lacks the cyclohexyl side chain (Des-CyH 506BD).[8] On the other hand, minor

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modifications of the side chain at position 21 have little effect on binding to FKBP (e.g., FK-506 vs ascomycin),[17] suggesting that this portion of the molecule may not be involved in binding to the protein. This is consistent with the lack of NOEs between the ethyl side chain of ascomycin and FKBP. It is interesting to note that those portions of ascomycin that were found to be in close proximity to FKBP from the NOE data are the same parts of the molecule in common with rapamycin, a potent immunosuppressant which also binds tightly to FKBP. Indeed, as noted previously,[14] the piperidine ring of rapamycin and FK-506 is involved in binding to FKBP and is likely to be oriented in a similar manner in the two ligand/receptor complexes.

Conclusions. From an analysis of NOEs observed in heteronuclear 3D NOE spectra of the [U-^{13}C]ascomycin/FKBP complex, the conformation of ascomycin when bound to its putative target protein, FKBP, was determined. The conformation of ascomycin was found to be very different from the X-ray structure of FK-506 determined in the absence of FKBP, but more closely resembles that of uncomplexed rapamycin. In addition, from the observation of ascomycin/FKBP NOEs in the 3D spectra, those portions of ascomycin that are in close proximity to FKBP were identified, and they include parts of the piperidine, pyranose, and cyclohexyl rings. By using the structural information obtained from this study it may be possible to design ascomycin analogues that are structurally dissimilar from ascomycin but which nonetheless maintain the necessary functionality in the proper orientation for binding to FKBP. It is hoped that analogues could be designed with improved physicochemical properties and potentially lower toxicity, thus making them superior immunosuppressive agents.

During the preparation of this manuscript, a report on the X-ray crystal structure of the FK-506/FKBP complex appeared.[23] The conformation of ascomycin when bound to FKBP as determined by NMR is very similar to the conformation of FK-506 when bound to FKBP as determined by X-ray crystallography. In both structures of the bound ligands, the 9,10 amide bonds are trans and the relative orientation of the pyranose and cyclohexyl rings are the same. In the X-ray structure roughly 50% of the ligand surface is buried at the protein–ligand interface with the region around the allyl and cyclohexyl groups being exposed to solvent. We observed no NOEs from the ethyl group of ascomycin (analogous to the allyl group in FK-506) to the protein, which is consistent with this portion of the molecule being exposed to solvent. However, we do observe several NOEs from the cyclohexyl ring (Figure 5) to the protein, which clearly indicates that a portion of this ring is in close proximity to the protein in solution.

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Supplementary Material Available: A table of ^{1}H and ^{13}C chemical shifts of [U-^{13}C]ascomycin bound to FKBP (1 page). Ordering information is given on any current masthead page. The coordinates of ascomycin when bound to FKBP will be deposited in the Brookhaven Protein Data Bank.

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Substituted 2-(Aminomethyl)piperidines: A Novel Class of Selective Protein Kinase C Inhibitors

Since its discovery in 1977, the ubiquitous enzyme protein kinase C (PKC)[1] has received extensive pharmacological investigation and has emerged as a pivotal mediator in cellular regulation, signal transduction, and neoplastic promotion. Physiological activity of the enzyme is regulated by the allosteric modulators Ca^{2+}, diacylglycerol (DAG), and phosphatidylserine (PS).[1,2] These modulators interact with the enzyme's regulatory domain while the catalytic domain possesses the site of ATP and substrate binding.[3] Phorbol esters have been found to substitute for diacylglycerol as a potent enzyme stimulator,[4] and in animals, phorbol ester activation of PKC causes intense inflammation. Unlike diacylglycerol, phorbol esters are not rapidly metabolized and thus can effect prolonged enzyme stimulation often leading to neoplastic events.[5]

Given the biological responses induced by activators of protein kinase C, the development of inhibitors of this enzyme may lead to therapeutic agents useful in the treatment of chronic inflammatory and proliferative diseases. Several natural and synthetic agents have been identified as PKC antagonists. These include (1) nonselective phospholipid competing agents such as triphenylethylenes,[6] chlorpromazine,[7] and trifluoperazine,[8] (2)

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