Brief Articles

Retention of Immunosuppressant Activity in an Ascomycin Analogue Lacking a Hydrogen-Bonding Interaction with FKBP12

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C24-Deoxyascomycin was prepared in a two-step process from ascomycin and evaluated for its immunosuppressant activity relative to ascomycin and FK506. An intermediate in the synthetic pathway, $\Delta^{23,24}$-dehydroascomycin, was likewise evaluated. Despite lacking the hydrogen-bonding interactions associated with the C24-hydroxyl moiety of ascomycin, C24-deoxyascomycin was found to be equipotent to the parent compound both in its immunosuppressive potency and in its interaction with the immunophilin, FKBP12. Conversely, $\Delta^{23,24}$-dehydroascomycin which also lacks the same hydrogen-bonding interactions did not exhibit this potency. NMR studies were conducted on the FKBP12/C24-deoxyascomycin complex in an attempt to understand this phenomenon at the molecular level. The NMR structures of the complexes formed between FKBP12 and ascomcyin or C24-deoxyascomcyin were very similar, suggesting that hydrogen-bonding interactions with the C24 hydroxyl moiety are not important for complex formation.

Introduction

Through the development and use of the cyclic peptide cyclosporin A, immunosuppressive therapy has been effective in the prevention of acute organ rejection following transplantation procedures and shows the potential to control autoimmune and inflammatory diseases.[1] In addition, the discovery of FK506, found while screening fermentation broths for interleukin 2 production inhibitors, heightened interest in this therapeutic arena by initiating entry into a novel second structural class of compounds having a 10−100-fold enhancement of immunosuppressive potency.[2,3] A necessary first step in FK506 action is binding to a *cis*−*trans* peptidyl−prolyl isomerase, the FK506 binding protein (FKBP12). The FK506/FKBP12 complex inhibits a serine/threonine phosphatase, calcineurin, the inhibition of which ultimately prevents transcriptional processes leading to interleukin 2 production from occurring within the T cell nucleus.[4] Three-dimensional structures of FKBP12 alone and when complexed to FK506 and FK506 analogues have been determined by X-ray crystallography and NMR spectroscopy.[5] Ascomycin, the analogue of interest for this report with in vitro potency comparable to that of FK506,[6] differs from FK506 only at the C21 substituent in which an ethyl group replaces an allyl side chain. The FKBP12/FK506 complex is stabilized by a series of hydrophobic interactions and intermolecular hydrogen bonds. Among the intermolecular hydrogen bonds found in all FKBP12/ligand complexes are those involving the C24 hydroxyl moiety. Glutamic acid 54 and glutamine 53 hydrogen bond with this moiety directly or through a bridging water molecule, respectively. The location of the C24 hydroxyl group is of particular interest in that it is found at, or near, the border between the sites that interact with FKBP12 and calcineurin.[7] In addition, there is recent speculation that this hydroxyl group is a hydrogen bond donor that mimics the NH group of the peptide substrate.[8]

As part of a continuing effort to probe the structure−activity relationships of these macrolactam immunosuppressants,[9] the importance of the hydrogen bonds involving the C24 hydroxyl group for the formation of the FKBP12/ascomycin complex was examined by comparison with its C24-deoxy analogue and the synthetic precursor to the deoxy analogue, $\Delta^{23,24}$-dehydroascomycin. In this paper, the synthesis, biological activity, and NMR studies of C24-deoxyascomycin are reported.

Results and Discussion

Synthesis of C24-Deoxyascomycin. Ascomycin was obtained as a fermentation product from *Streptomyces hygroscopicus* var. *ascomyceticus* by methods similar to those described in the literature.[10] The synthesis of C24-deoxyascomycin was accomplished from ascomcyin by a variation of a two-step process (Scheme 1).[11] Transformation to the $\Delta^{23,24}$-enone was selectively and efficiently achieved by using *p*-toluenesulfonic acid in toluene at 75−80 °C. Evidence for formation of the dehydration product, which like ascomycin and FK506 exists as a mixture of amide rotamers, was obtained from the ^{13}C NMR spectrum of the product in which new signals at 147.8/146.2 (C24) and 129.0/127.6 (C23) ppm corresponding to the α and β carbons of the enone system were observed. Reduction of the

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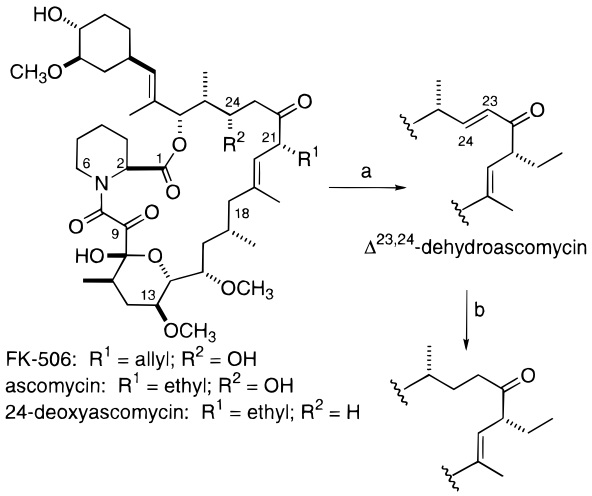
Scheme 1[a]



[a] Reagents: (a) *p*-TsOH·H_2O, toluene, reflux; (b) H_2, Pd−C, MeOH.

Table 1. Biological Evaluation of Ascomycin and Ascomycin Analogues

	IC_{50} (nM)		
compound	FKBP12 binding	MLR potency	PLN potency ED_{50} (mg/kg/day IP)
FK506	2.3	0.23	0.15
ascomycin	2.1	0.26	0.32
$\Delta^{23,24}$-dehydroascomycin	529	22	39%[a]
C24-deoxyascomycin	0.77	0.15	0.65

[a] Percent response of $\Delta^{23,24}$-dehydroascomycin at 3 mg/kg/day ip.

$\Delta^{23,24}$-enone was then accomplished by exposure to hydrogen gas in the presence of palladium on activated carbon at room temperature. The ^{13}C and ^{1}H NMR spectra obtained were consistent with the reduction of the enone moiety and retention of the two trisubstituted olefins.

Pharmacology. To examine the binding affinity of the FK506 analogues to FKBP12, a binding assay was utilized in which the analogue competes with labeled ascomycin for binding to an FKBP12−CMP−KDO synthetase fusion protein.[12] As shown in Table 1, FK506, ascomycin, and C24-deoxyascomycin all bind comparably. The lone standout in this assay is $\Delta^{23,24}$-dehydroascomycin which is several hundred times less potent. Although this may be explained in part by the loss of the hydrogen bonds involving the C24 hydroxyl moiety, the decreased affinity may also be due to conformational changes caused by the introduction of two new sp^2 hybridized centers. Surprisingly, reintroduction of sp^3 hybridization, without the hydrogen bonding, results in total restoration of the interaction with FKBP12.

The immunosuppressive activities of these compounds were examined in an in vitro model of allogeneic T lymphocyte activation (MLR)[3] as well as in an in vivo model of lympho-proliferation, the popliteal lymph node (PLN)[13] assay. In the MLR assay, human peripheral blood mononuclear leukocytes (PBL) were exposed to a pool of mitomycin C-treated PBL. Four days hence, the incorporation of [^{3}H]thymidine into cellular DNA was measured by liquid scintillation spectrometry. Complete inhibition of this response was observed for FK506, ascomycin, and C24-deoxyascomycin. By contrast, $\Delta^{23,24}$-dehydroascomycin, reflective of its poor interaction with FKBP12, was approximately 2 orders of magnitude less potent than the other compounds (Table 1). In the second assay in which the PLN hyperplasia response to alloimmune challenge with histoincompatible splenocytes was measured, the results were similar to those found for the in vitro experiment. Ascomycin, FK506, and C24-deoxyascomycin exhibited similar potencies, whereas $\Delta^{23,24}$-dehydroascomycin did not give a significant response (Table 1).

NMR Studies of the FKBP12/C24-Deoxyascomycin Complex. The tight binding affinity and high immunosuppressive potency of C24-deoxyascomycin was surprising since two of the five hydrogen bonds in the FKBP12/ascomycin complex cannot be formed in the FKBP complex with the C24 deoxy analogue. In an attempt to understand this phenomenon at the molecular level, NMR studies were conducted on the FKBP12/C24-deoxyascomycin complex.

As a first step, the conformation of C24-deoxyascomycin when bound to FKBP12 was determined from isotope-filtered NMR experiments. In an isotope-filtered 2D TOCSY spectrum, the scalar-coupled protons were readily traced. This information coupled with an analysis of the isotope-filtered 2D NOE spectrum allowed the ^1H NMR signals corresponding to C24-deoxyascomycin when bound to FKBP12 to be assigned. To determine the conformation of C24-deoxyascomycin when bound to FKBP12, 126 intraligand NOEs were assigned from the isotope-filtered 2D NOE spectrum of the complex. Proton−proton distance restraints were generated from the NOE data, and 200 structures were generated that were consistent with the distance restraints using a dynamical simulated annealing protocol.[14] A total of 62 low-energy structures were chosen from this set that best satisfied the NOE restraints. The structure of the ligand when bound to FKBP was very well-defined by the NMR data and deviated only slightly from idealized geometry. The average rms deviation of all heavy atoms from a calculated average structure was 0.28 ± 0.11 Å, and no NOE violations were found to be greater than 0.1 Å (see Supporting Information).

As shown in Figure 1, the bound conformation of C24-deoxyascomycin was found to be very similar to that of ascomycin when bound to FKBP12.[5d] Superposition of the common heavy atoms of the C24-deoxyascomycin analogue and ascomycin gives an rms deviation of 0.35 Å. This is consistent with the similar chemical shifts observed for ascomycin and C24-deoxyascomycin. Except in the immediate vicinity of C24, the chemical shifts of the analogue were very similar to ascomycin, including the characteristic upfield proton resonances of the 3, 4, and 5 positions of the piperidine ring.[15] Thus based on chemical shifts, the overall structure of the complex must be similar despite the lack of the two hydrogen bonds involving the C24−OH in ascomycin.

Although the conformations of ascomycin and the C24 deoxy analogue are similar, it is possible that the ligands interact with the protein in a different manner. Thus, the backbone chemical shifts of FKBP were compared while bound to the two ligands. As shown in Figure 2, the substitution of a proton for the OH functional group of ascomycin results in significant changes in chemical shift of the protein all along the

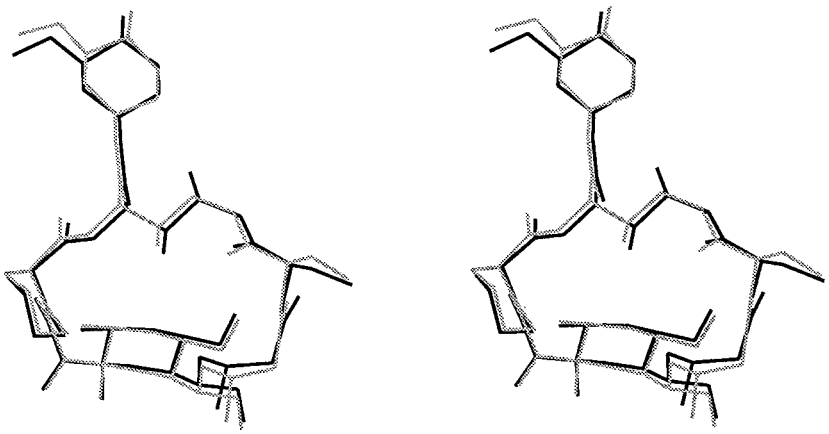


Figure 1. Superposition of the average, minimized structure of C24-deoxyascomycin bound to FKBP (gray) to the average, minimized structure of ascomycin bound to FKBP (black).

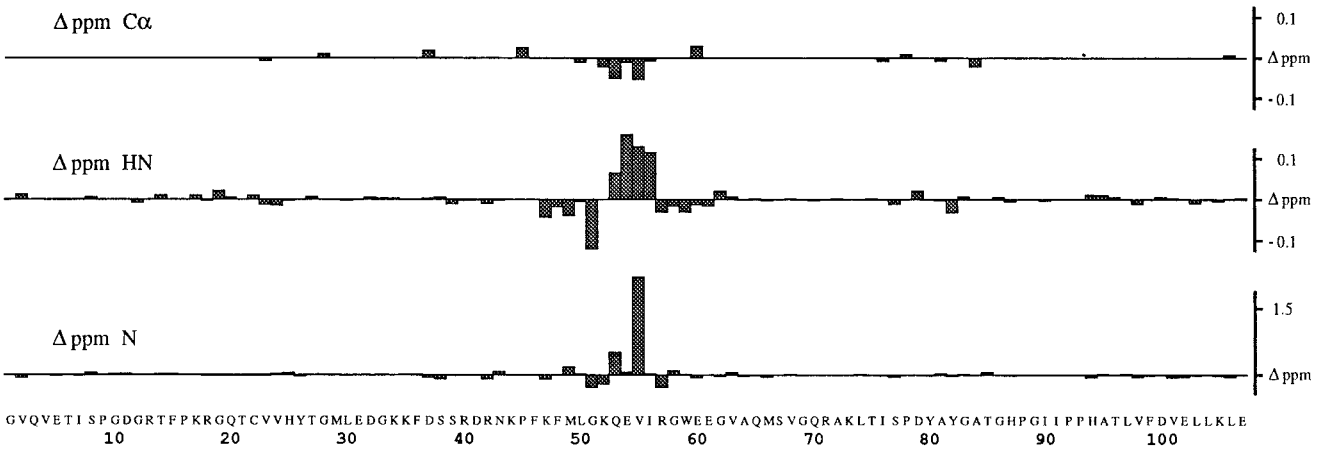


Figure 2. Analysis of chemical shift differences observed between the two FKBP12 complexes. The shifts were obtained by subtracting the chemical shifts of the $^1H^{\alpha}$, $^1H^N$, and ^{15}N resonances of [U-^{15}N,^{13}C]FKBP12 ligated to unlabeled C24-deoxyascomycin from the corresponding resonances of [U-^{15}N,^{13}C]FKBP12 ligated to unlabeled ascomycin. The scale of the ^1H shift differences is not the same as that of the ^{15}N differences.

loop from Gly-51 to Ile-56, the region of the protein closest to the site of modification on the ligand. Most of these changes are relatively small and may simply be related to the presence or absence of the oxygen at C24. However, an unusually large ^{15}N chemical shift difference of 2.2 ppm was observed for the Val-55. A likely cause of this large chemical shift difference is the direct effect of the hydrogen bond to the Glu-54 carbonyl. In the FKBP12/ascomycin complex, the hydrogen bond between the C24−OH of ascomycin and the Glu-54 carbonyl of FKBP12 stabilizes the amide bond resonance structure in which there is increased double-bond character between nitrogen and carbon[16] resulting in a decrease of the electron density on the nitrogen atom of Val-55 and a downfield shift relative to the C24 deoxy analogue.

To further investigate possible differences in the conformation of FKBP12 when bound to the two ligands, a 4D [^{13}C,^1H,^{15}N,^1H] NOESY and a 4D [^{13}C,^1H,^{13}C,^1H] NOESY was recorded on both complexes. Two 2D ω_1, ω_2 slices from the 4D [^{13}C,^1H,^{15}N,^1H] NOESY spectra at the same ^{15}N (ω_3) and ^1H (ω_4) amide frequencies of Gln-53 and Glu-54 in both complexes were compared. These amides are located near the two hydrogen bonds observed in the FKBP/ligand complex. The NOEs are nearly identical in the spectra of the two complexes. Similarly, the 4D [^{13}C,^1H,^{13}C,^1H] NOE data is the same in the two complexes (see Supporting Information). Thus the NOE data indicates that the complexes formed between FKBP12 and ascomycin and the C24 deoxy analogue are structurally very similar.

Conclusions

In the three-dimensional structure of the FKBP12/FK506 complex, two of the five intermolecular hydrogen bonds involve the C24 hydroxyl group. One hydrogen bond is formed directly between the Glu-54 carbonyl and the C24 hydroxyl group, and the second, mediated by a water molecule, involves Gln-53. Despite the absence of these hydrogen bonds between the ligand and the protein in the FKBP12/C24-deoxyascomycin complex, the C24 deoxy analogue interacts as strongly to FKBP12 as does ascomycin in a competitive binding assay. In addition, both molecules display similar immunosuppressant activities. The conformations of the bound ligands are similar as are the overall structures of the FKBP/ligand complexes. These observations suggest that the hydrogen bonds involving the C24 hydroxyl group do not significantly enhance the binding of ascomycin to the protein. One possible explanation is that upon hydrogen bond formation, a loss of entropy is obtained by decreasing the motion of an otherwise mobile loop of FKBP12 and confining the location of a water molecule to mediate one of the hydrogen bonds.

Both effects may lead to an unfavorable entropic process that compensates to a large degree any favorable enthalpic reaction that might be gained upon forming hydrogen bonds. The major driving force for complex formation may be hydrophobic in nature. Indeed, FK506 binds in a hydrophobic pocket lined with nonpolar groups. The ligands themselves contain many nonpolar groups and are quite insoluble in water. The loss of two hydrogen bonds appears not to be as significant to the complex formation as was originally assumed.

Experimental Section

Ascomycin and FK506 were produced by fermentation using procedures similar to those that have been previously described in the literature.[10] Unless otherwise indicated, reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin−streptomycin was purchased from Gibco Laboratories (Grand Island, NY). Complete RPMI 1640 contained 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. FK506 binding protein (FKBP12) and the fusion protein composed of FKBP12 and CMP−KDO synthetase (FKBP12/CKS) were prepared at Abbott Laboratories (Abbott Park, IL) as previously described.[12] PMBCs were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from healthy donors. Stimulator PBMCs were treated with 25 μg/mL mitomycin C for 30 min and washed before being used.

Chemistry. General Methods. See Supporting Information. Synthesis of $\Delta^{23,24}$-Dehydroascomycin. Ascomycin (6.00 g, 7.58 mmol) was dissolved in toluene (120 mL) and the solution heated to 75−80 °C. *p*-Toluenesulfonic acid monohydrate (300 mg) was introduced in one portion. After 1 h, the reaction was incomplete and additional *p*-toluenesulfonic acid monohydrate (300 mg) was added. Thirty minutes later, the reaction was complete, and the mixture was cooled to room temperature. Without concentration, the reaction solution was passed through a plug of silica gel (75 g) eluting with ether (1000 mL). Concentration of the eluent in vacuo provided 5.47 g of crude product as a yellow solid. Purification by flash chromatography (silica gel, elution with 35% acetone in hexane) furnished the title compound as a colorless solid (3.16 g, 54%): mp 122−124 °C; ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.2 and 199.6 (C22), 196.0 and 191.4 (C9), 169.2 and168.8 (C1), 165.9 and 165.1 (C8), 147.8 and 146.2 (C24), 139.1 and 138.0 (C19), 133.2 and 131.7 (C27), 131.1 and 130.0 (C28), 129.0 and 127.6 (C23), 123.9 and 123.4 (C20), 98.7 and 97.9 (C10), 84.1 (C31), 80.4 and 79.6 (C26), 76.0 and 75.8 (C15), 74.1 and 73.8 (C32), 73.5 and 73.5 (C13−OMe), 73.4 and 71.6 (C14), 57.5 and 56.2 (C15−OMe), 56.8 (C2), 56.6 (C13−OMe), 56.4 (C31−OMe), 53.6 and 53.3 (C21), 49.1 and 48.8 (C18), 39.8 and 39.1 (C25), 38.4 and 33.7 (C6), 35.7 and 35.0 (C29), 34.9 and 34.7 (C11), 34.6 and 34.4 (C30), 33.0 and 32.7 (C16), 32.4 (C12), 31.2 (C33), 30.6 and 30.5 (C34), 27.9 and 27.4 (C3), 26.5 and 26.4 (C17), 25.5 and 25.4 (C35), 24.7 and 24.2 (C5), 21.0 (C17-Me), 20.3 and 18.9 (C4), 16.1 and 15.9 (C11-Me), 14.5 and 14.2 (C27-Me), 12.9 and 12.5 (C36), 11.8 and 11.7 (C25-Me); MS (CI) *m*/*z* 791 (M + NH_4^+); IR (KBr) cm^{-1} 3440, 2930, 1745, 1690, 1645, 1625, 1450, 1390, 1242, 1190, 1170, 1100. Anal. ($C_{43}H_{67}NO_{11}$) C, H, N.

Synthesis of C24-Deoxyascomycin. $\Delta^{23,24}$-Dehydroascomycin (1.00 g, 1.29 mmol) was added to a suspension of 10% palladium on carbon (100 mg) in methanol. The reaction vessel was evacuated and filled with nitrogen several times before being placed under a hydrogen atmosphere (balloon). The reaction mixture was vigorously stirred with a magnetic stirrer for 1.5 h. The catalyst was removed by filtration through Celite and rinsed with methanol. The filtrate was concentrated in vacuo to supply 0.93 g (93%) of the desired product as a colorless foam. Analytically pure material was obtained by flash column chromatography (silica gel, elution with 25% acetone in hexane) although a loss in product was encountered (0.35 g, 35%): ^{13}C NMR (125 MHz, $CDCl_3$) δ 212.2, 210.8, 196.3, 193.4, 169.4, 169.3, 166.1, 165.1, 138.9, 138.3, 132.1, 131.6, 131.1, 124.4, 124.1, 98.4, 97.4, 84.2, 82.3, 82.2, 76.8, 75.5, 73.8, 73.5, 73.5, 73.1, 72.4, 57.2, 57.1, 56.5, 56.3, 56.2, 55.4, 54.7, 52.6, 49.3, 48.4, 43.8, 39.2, 39.1, 38.3, 35.3, 34.9, 34.9, 34.7, 34.6, 34.3, 33.3, 32.7, 31.2, 30.6, 28.6, 27.7, 26.5, 26.3, 26.1, 24.5, 24.3, 24.0, 20.9, 20.7, 19.9, 19.3, 16.1, 16.0, 15.7, 15.5, 14.9, 14.4, 13.7, 13.5, 11.7; MS (FAB + KI) *m*/*z* 814 (M + K^+); IR (KBr) cm^{-1} 3440, 2940, 1742, 1715, 1650, 1455, 1382, 1198, 1175, 1100. Anal. ($C_{43}H_{69}NO_{11}$) C, H, N.

Biological Methods. FKBP12 binding assay: Human FKBP12 was cloned as a fusion partner with CMP−KDO synthetase (CKS) and purified as described by Edalji et al.[12] An ascomycin conjugate of alkaline phosphatase was prepared by active ester coupling of an ascomycin C22 derivative (carboxymethyloxime) with alkaline phosphatase (ascomycin-AP; Abbott Laboratories, Abbott Park, IL). In the assay, FKBP12−CKS fusion protein was dissolved in 10−35 μg/mL in 20 mM sodium phosphate buffer, pH 7.4, and adsorbed to the wells of an Immuno Plate Maxisorp (Nunc, Naperville, IL) by incubation at ambient temperature for 2 h. A solution of phosphate-buffered saline (PBS), pH 7.4, containing 2% bovine serum albumin (BSA) and 0.2% Tween 20 was added to the wells. After rinsing the wells with 0.2% Tween 20 in PBS, the test compound in the PBS/BSA/Tween 20 buffer, or buffer alone, was added to the wells. An equal volume of ascomycin-AP ligand at 1 μg/mL in PBS/BSA/Tween 20 was added to the wells and incubated 2 h at ambient temperature. After rinsing with 0.2% Tween 20 in PBS, *p*-nitrophenyl phosphate at 1 mg/mL in 0.1 M aminomethylpropanol was added to the wells and the temporal change in 405-nm absorbance recorded.

Human mixed leukocyte response: One-way allogeneic mixed leukocyte response assays were performed as described by Kino et al. with slight modification.[3] Briefly, 1×10^5 responder PBMCs were mixed with 4×10^5 stimulator PBMCs, composed of 1×10^5 PBMCs from each of four different donors, in 0.2 mL of complete RPMI 1640 and cultured for 96 h at 37 °C. During the final 6 h, the cells were labeled with 0.5 μCi/well of tritiated thymidine ([^{3}H]TdR; DuPont NEN Research Products, Boston, MA). The cells were harvested by vacuum filtration onto glass fiber filters, and the radioactivity was measured by liquid scintillation spectrometry. Test compounds, dissolved at 10 μM in dimethyl sulfoxide, were diluted in complete RPMI 1640 and added to the responder PBMCs before addition of the stimulator PBMCs.

PLN hyperplasia assay: Inbred male rats (125−150 g) were purchased from Harlan Sprague−Dawley, Inc. (Indianapolis, IN) and housed for 1 week before use. Spleen cells from Brown Norway rats (RT-1^n) were subjected to ammonium chloride to lyse red cells, washed in phosphate-buffered saline (PBS), X-irradiated (2000 rads), washed again, and injected subcutaneously with a 27-gauge needle in the right hind foot of Lewis strain rats (RT-1^l) using 5×10^6/100 μL. Test compound was dissolved in a vehicle consisting of 10% ethanol, 40% propylene glycol, and 2% cremophore with the balance being sterile 5% dextrose in water (Abbott Laboratories, Abbott Park, IL); 2 mL/kg of the test compound solution was given ip to groups of 8 animals once daily, on days 0−3. On day 4, the PLN were removed and weighed on a microbalance. The mean weight of nodes from a group of uninjected control animals was subtracted to determine the net increase, and percent inhibition in reference to a vehicle control group was calculated.

Statistical analysis: Unless otherwise indicated, all data are presented as the mean ± SEM and were analyzed using Student's *t*-test of unpaired samples.

NMR sample preparation for FKBP/ligand complexes: Recombinant human FKBP was cloned from a Jurkat T-cell cDNA library and expressed at high levels in *Escherichia coli* using translational coupling to the 5′ end of the *E. coli kdsB* gene. [U-^{13}C,^{15}N]FKBP were purified from cells grown on minimal medium containing [^{15}N]ammonium chloride and [U-^{13}C]acetate[17] using ion-exchange and size-exclusion chromatography.[12] The protein was concentrated to about 3 mM

and exchanged into either an H_2O or D_2O solution (pH 6.5) containing potassium phosphate (50 mM), sodium chloride (100 mM), and dithiothreitol-d_{10} (5 mM). FKBP12/ligand complexes (1/1) were prepared by incubating labeled FKBP12 with an excess amount of either unlabeled ascomycin or unlabeled C24-deoxyascomycin for 24−48 h at room temperature.

NMR experiments: All NMR spectra of the FKBP/ligand complexes were recorded on either a Bruker AMX500 (500 MHz) or an AMX600 (600 MHz) spectrometer. NMR spectra were processed and analyzed using in-house written software on Silicon Graphics computers. 2D isotope-filtered NOESY and TOCSY spectra of the unlabeled ligand while bound to [U-^{13}C,^{15}N]FKBP were recorded at 40 °C using pulse sequences described earlier.[18] Mixing times of 80 and 15 ms were used for the NOESY and TOCSY experiments, respectively.

4D NOESY spectra of [U-^{13}C,^{15}N]FKBP while complexed with unlabeled ligand were recorded at 30 °C. The 4D [^{13}C,^{1}H,^{15}N,^{1}H]NOESY spectrum[19] was acquired with eight scans per experiment with 12 × 80 × 10 × 512 complex points using sweep widths of 3289, 7463, 2128, and 10 000 Hz in ω_1(^{13}C), ω_2(^{1}H), ω_3(^{13}C), and ω_4(^{1}H), respectively. Water suppression was accomplished[20] using two spin−lock pulses of 0.5- and 2.5-ms duration. The ^{13}C carrier frequency was set at 38 ppm. The regions from 70−49 and 27−5.5 ppm were folded once. The 4D [^{13}C,^{1}H,^{13}C,^{1}H] NOESY spectrum[21] was acquired with eight scans per experiment with 12 × 64 × 12 × 512 complex points using sweep widths of 3289, 5208, 3289, and 10 000 Hz in ω_1(^{13}C), ω_2(^{1}H), ω_3 (^{13}C), and ω_4 (^{1}H), respectively. The ^{13}C spectral widths were folded in the same manner as in the 4D [^{13}C,^{1}H,^{15}N,^{1}H] NOESY experiment. The ^{1}H carrier was centered at 3.7 ppm. In both 4D experiments, a 10-ms homospoil was applied during the 50-ms NOESY mixing time to suppress unwanted magnetization, and a series of randomly spaced 90° pulses were used to saturate the ^{13}C spins at the beginning of the pulse sequence.[21b]

NOE-derived distance restraints and structure calculations: The proton−proton distances used as restraints for the structure calculations of the C24 deoxy derivative of ascomycin were obtained by counting contours in the isotope-filtered NOESY data set collected with a mixing time of 80 ms. NOEs were classified as either strong (1.8−2.8 Å), medium (1.8−3.4 Å), or weak (1.8−4.4 Å). For distances involving methyl groups, 1.0 Å was added to the upper bound to correct for pseudoatom.[22] A total of 126 NOE restraints were used along with 5 lower bound restraints that were determined on the basis of the lack of observable NOEs.

3D structures were calculated using a hybrid distance geometry/dynamical simulated annealing protocol.[14] Using the XPLOR[23] program and the NOE-derived distance restraints, 200 initial structures were generated and subjected to 200 steps of Powell restrained energy minimization to remove bad van der Waals contacts. During this minimization, and throughout the entire simulated annealing protocol, the NOE force constant was maintained at 50 kcal·mol^{-1}·Å^{-2}, and electrostatic terms were excluded. The minimization step was followed by 7.5 ps of molecular dynamics (time-step of 3 fs) at 2000 K during which the van der Waals force constant was decreased from its initial value of 20 kcal·mol^{-1}·Å^{-2} to a value of 0.003 kcal·mol^{-1}·Å^{-2} while increasing all other force constants (bond, angle, etc.). The structures were then cooled from 2000 to 100 K in steps of 50 K. Each step of the cooling process consisted of 1.25 ps of restrained molecular dynamics (time-step of 5 fs). The van der Waals force constant was increased at each step by multiplying the previous value by 1.28 until a final value of 4.0 kcal·mol^{-1}·Å^{-2} was obtained. The van der Waals radius was decreased stepwise to a final value of 0.8 times the value used in CHARM for Frepel.[24] In the last stage of the refinement, the structures were subjected to 1000 steps of Powell restrained energy minimization.

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Supporting Information Available: Isotope-filtered 2D TOCSY and NOESY spectra and structural statistics for C24-deoxyascomycin bound to [U-^{13}C,^{15}N]FKBP; ^{13}C(ω_1), ^{1}H(ω_2) planes from the 4D [^{13}C,^{1}H,^{15}N,^{1}H] NOESY spectrum of [U-^{15}N,^{13}C]FKBP12 complexed with ascomycin or 24-deoxyascomycin extracted at the ^{1}H^{N},^{15}N chemical shifts of the individual residues Glu-54 and Gln-53, respectively; ^{13}C(ω_1), ^{1}H(ω_2) planes from the 4D [^{13}C,^{1}H,^{13}C,^{1}H] NOESY spectrum of ligated [U-^{15}N,^{13}C]FKBP12 extracted at the ^{1}H,^{13}C chemical shifts of Arg-57 H$^{\delta 2}$ and Val-55 H$^{\gamma 1}$, respectively; chemistry general methods. This information is available free of charge via the Internet at http://pubs.acs.org.

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