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Conformation of two non-immunosuppressive FK506 analogs when bound to FKBP by isotope-filtered NMR

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The 3D structure of two unlabeled FK506 analogs, (*R*)- and (*S*)-[18-OH]ascomycin, when bound to [U-^{13}C,^{15}N]FKBP were determined by isotope-filtered 2D NMR experiments. The structures for the *R* and *S* isomers that bind tightly to FKBP but lack immunosuppressive activity are compared to each other and to the conformation of the potent immunosuppressant, ascomycin, when bound to FKBP. The results are interpreted in terms of calcineurin binding to the FKBP/ascomycin complex.

FKBP; FK506; Immunosuppressant; NMR

1. INTRODUCTION

FK506 is a potent immunosuppressant that binds tightly (K_d ~0.4 nM) to the FK506 binding protein (FKBP-12) [1,2]. Recent studies suggest that the immunosuppressive activitiy of FK506 is due to the inhibition of the calcium-dependent phosphatase, calcineurin, by the FK506/FKBP complex [3]. In an attempt to better understand the structural features required for the inhibition of calcineurin by this complex, we have synthesized several FK506 analogs and have tested their ability to bind to FKBP, and, once bound, their ability to inhibit calcineurin [4]. These analogs are derivatives of ascomycin [5], also known as FR-900520 [6], which is a potent immunosuppressant closely related to FK506 in structure and activity (Fig. 1). Two analogs were discovered, *R*- and *S*-[18-OH]ascomycin, that bind tightly to FKBP but have little or no immunosupressive activity [4,7]. In addition, the complexes formed between these analogs and FKBP did not inhibit the phosphatase activity of calcineurin [4,7]. In order to probe the structural features of these ascomycin analogs that might account for their interesting biological properties, we have examined their conformation when bound to FKBP.

Previously, we determined the conformation of [U-^{13}C]ascomycin bound to FKBP via isotope-editing techniques [8,9]. Using these methods the signals from the unlabeled protein are suppressed, and only those signals from the bound ligand are observed [10–13]. However, we could not apply these techniques in our studies of the 18-OH analogs due to the difficulties in preparing the uniformly ^{13}C-labeled ligands. Therefore, we resorted to recently developed isotope-filtering techniques [14–16] in which the proton NMR signals corresponding to uniformly ^{13}C-labeled FKBP were effectively suppressed using a doubly tuned filter [15] to allow the selective detection of unlabeled (*R*)- and (*S*)-[18-OH]ascomycin when bound to FKBP. These methods offer a powerful alternative to isotope-editing techniques for studying drug/receptor complexes, since they do not require isotopically labeled ligands, which in most cases cannot be readily obtained.

Abbreviations: NOE, nuclear Overhauser effect; FKBP, FK506 binding protein; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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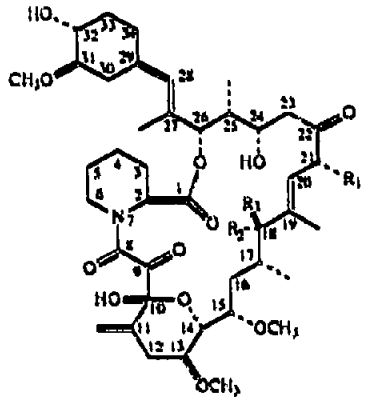
2. MATERIALS AND METHODS

2.1. *Sample preparation*

Synthesis of the [18-OH]ascomycin derivatives, via oxidation of ascomycin by selenium (IV) oxide, will be described in detail elsewhere [4]. Recombinant human FKBP-12 was cloned from a Jurkat T cell cDNA library and expressed in *E. coli* using the pKK233-2 vector containing a trc promoter [17]. [U-^{13}C,^{15}N]FKBP was prepared by growing the FKBP-producing cells on minimal media with [U-^{13}C]acetate as the sole carbon source and $^{15}NH_4Cl$ as the sole nitrogen source, and was isolated from these cells using ion-exchange and size-exclusion chromatography [17]. NMR samples were prepared by exchanging the protein into a ^2H_2O solution (pH 6.5) containing potassium phosphate (50 mM), sodium chloride (100 mM), and dithiothreitol-d_{10} (5 mM), and concentrating the solution to ~500 μl using a centricon-10 microconcentrator. The final FKBP concentration was ~5 mM. The [18-OH]ascomycin/FKBP complexes were prepare d by incubating the protein solution overnight with an equimolar amount of each ligand.

2.2. *NMR experiments*

All NMR spectra were recorded on a Bruker AMX500 (500 MHZ)



	R_1	R_2	R_3
FK506	$CH_2CH{=}CH_2$	H	H
Ascomycin	CH_2CH_3	H	H
(*S*)-(18-OH)ascomycin	CH_2CH_3	OH	H
(*R*)-(18-OH)ascomycin	CH_2CH_3	H	OH

Fig. 1. Structures of FK506 and ascomycin.

spectrometer at 40°C. Isotope-filtered NOESY spectra, τ_m = 80 ms, were recorded in two different ways. The first method employed a conventional, υ_1, X half-filter [18] using the pulse sequence shown in Fig. 2A. The delay, Δ, was set at 3.6 ms. The second method employed a doubly-tuned, υ_1 filter [15] using the pulse sequence shown in Fig. 2B. The delays, Δ and Δ', were set at 4.00 and 3.56 ms, respectively, and spin-lock pulses (SL) of 1.00 and 1.50 ms duration were used. An isotope-filtered TOCSY spectrum was recorded, τ_m = 15 ms, with a doubly-tuned filter in both υ_1 and υ_2, using the pulse sequence shown in Fig. 2C. The delays, Δ and Δ', were set at 4.00 and 3.56 ms, respectively, and spin-lock pulses (SL) of 1.50, 1.00, 0.45 and 0.75 ms duration were used. Each 2D spectrum consisted of 256 complex t_1 points, with 64 scans per point and with a spectral width in both dimensions of 10,000 Hz. The final size for each data set was 4,096×1,024 real points.

2.3. *NOE-derived distance restraints and structure calculations*

For each derivative, the proton–proton distances used as restraints in the structure calculation were obtained by counting contours in the 80 ms NOESY data set recorded with the doubly-tuned filter. 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 the pseudoatom [19]. For (*S*)-[18-OH]ascomycin, a total of 111 NOE restraints were used along with 5 lower bound restraints that were determined on the basis of the lack of observable NOEs. For the *R* isomer, 117 NOE restraints and 5 lower bound restraints were used.

3D structures were calculated using a hybrid distance geometry/dynamical simulated annealing protocol [20]. Using the program XPLOR/DG [21,22] 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.

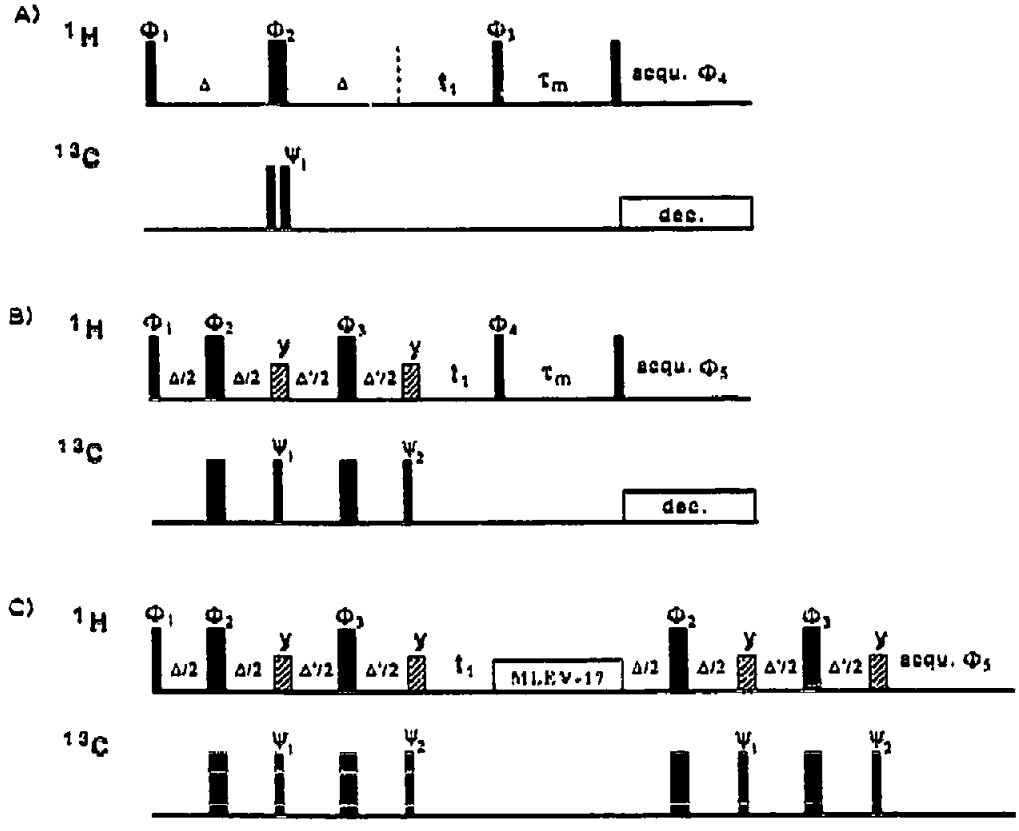


Fig. 2. Pulse sequences used to record isotope-filtered NOESY and TOCSY spectra. 90° pulses are represented by thin bars, 180° pulses by thick bars and spin-lock pulses by cross-hatched bars. All pulses are applied along the X-axis except as indicated otherwise. τ_m is the NOE mixing time. (A) NOESY sequence with conventional, (υ_1) X half-filter. Φ_1=4(x),4(−x); Φ_2=2(x),2(−x); Φ_3=8(x),8(−x); Φ_4=4(x),8(−x),4(x); ϑ_1=x,−x. (B) NOESY sequence with doubly-tuned (υ_1) filter. Φ_1=4(x),4(−x); Φ_2=x,−x; Φ_3=2(x),2(−x); Φ_4=8(x),8(−x); Φ_5=4(x),8(−x)4(x); ϑ_1=y,−x,−y,x; ϑ_2=x,y,−x,−y. (C) TOCSY sequence with doubly-tuned (υ_1,υ_3) filter. Φ_1=4(x),4(−x); Φ_2=x,−x; Φ_3=2(x),2(−x); Φ_4=4(x),4(−x); ϑ_1=y,−x,−y,x; ϑ_2=x,y,−x,−y.

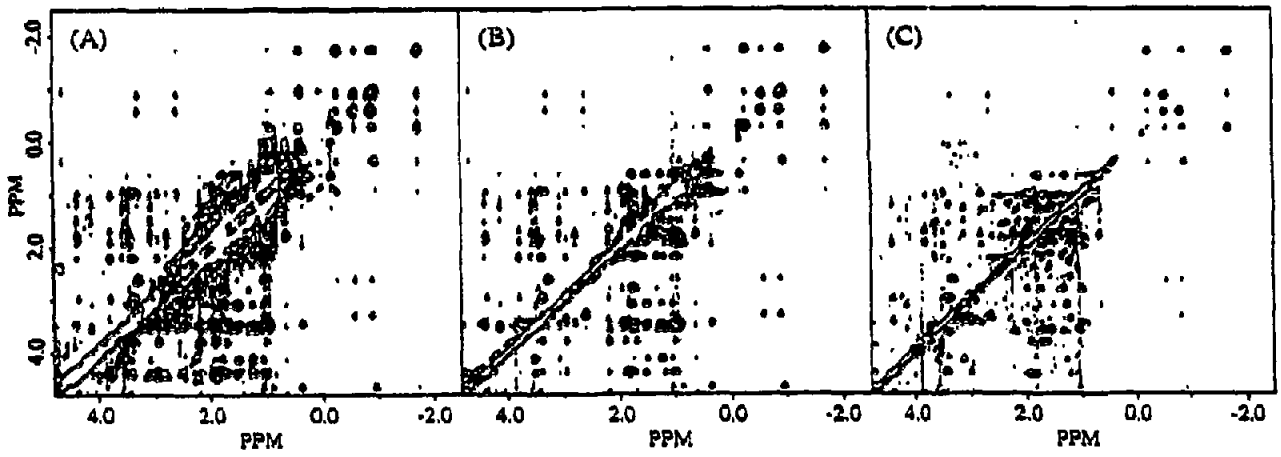


Fig. 3. Isotope-filtered spectra (500 MHz) recorded on the major stereoisomer of [18-OH]ascomycin bound to FKBP. (A) NOESY spectrum (τ_m = 80 ms) with conventional, (ω_1) X half-filter. (B) NOESY spectrum (τ_m = 80 ms) with doubly-tuned (ω_1) filter. (C) TOCSY spectrum (τ_{mix} = 15 ms) with doubly-tuned (ω_1,ω_2) filter.

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 2,000 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 2,000 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 CHARMM for F_{repel} [23]. In the last stage of the refinement, the structures were subjected to 1,000 steps of Powell restrained energy minimization.

3. RESULTS AND DISCUSSION

3.1. *Isotope-filtered NMR*

The selective observation of protons attached to ^{13}C-labeled ligands by suppressing the proton resonances of the unlabeled protein is relatively easy to achieve using conventional isotope-editing techniques [10–13,18]. However, to suppress the proton signals of ^{13}C-labeled protein and selectively observe the resonances of the unlabeld ligand is more difficult. The difficulties arise from the inability to simultaneously tune the fixed delays, Δ, in the conventional experiment to $1/(2\ ^1J_{H,}13_C)$ (Fig. 2A) for the different $^1J_{H,}13_C$ couplings typically

Table 1

Structural statistics for (*R*)- and (*S*)-[18-OH] ascomycin bound to FKBP

	(*R*)-[18OH] ascomycin		(*S*)-[18OH] ascomycin	
	SA_i	AV_m	SA_i	AV_m
rms deviation from distance restraints (Å)[a]	0.014±0.003	0.012	0.008±0.001	0.008
rms deviation from idealized geometry				
bonds (Å)	0.0080±0.0004	0.008	0.008±0.0004	0.0070
angles (deg)	2.72±0.083	2.61	2.55±0.037	2.52
impropers (deg)	0.64±0.05	0.62	0.62±0.03	0.59
Conformational Energy				
E_{NOE} (kcal/mol[b])	0.77±0.29	0.49	0.20±0.06	0.24
E_{repel} (kcal/mol)[c]	2.78±0.53	2.35	2.78±0.24	1.84
$E_{L,J}$ (kcal/mol)[d]	−3.28±0.44	−2.54	−3.18±0.78	−3.29
Atomic rms differences for all heavy atoms	SA_i vs. AV	0.28±0.11	SA_i vs. AV	0.37±0.07
	Sa_i vs. AV_m	0.37±0.12	SA_i vs. AV_m	0.45±0.19
	AV vs. AV_m	0.25	AV vs. AV_m	0.30

[a] SA_i is the average over the final 80 (*R*) or 85 (*S*) structure; AV is the average structure; AV_m is the structure obtained after restrained energy minimization of AV.

[b] E_{NOE} is the energy contribution from the square-well NOE potential using a force constant of 50 kcal/mol·Å^2.

[c] E_{repel} is the van der Waals repulsion enery term calculated with the F_{repel} potential and a force constant of 4 kcal·mol^{-1}·Å^{-2} and a hard-sphere van der Waals radii 0.8 times the CHARMM function.

[d] $E_{L,J}$ is the Lennard-Jones van der Waals energy calculated using the CHARMM function.

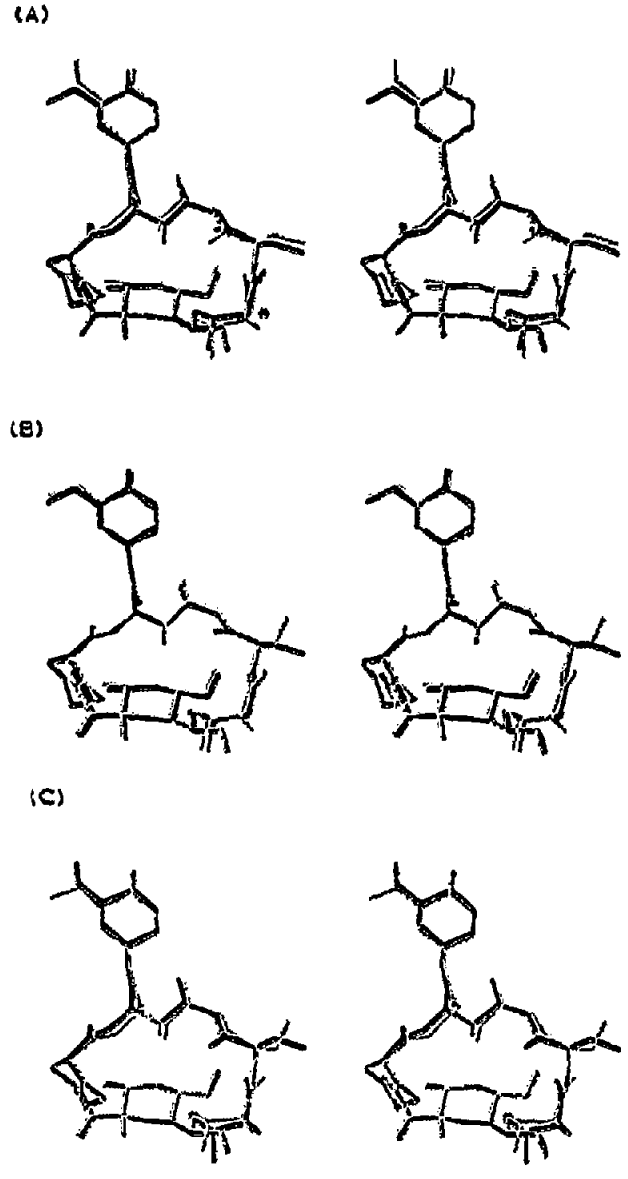


Fig. 4. (A) Superposition of the common heavy atoms of the average NMR structure of (*S*)-[18-OH]ascomycin bound to FKBP to the average NMR structure of ascomycin (bold) bound to FKBP. The 18 position is denoted with an asterisk. (B) Superposition of the common heavy atoms of the average NMR structure of (*R*)-[18-OH]ascomycin bound to FKBP to the average NMR structure of ascomycin bound to FKBP. (C) Superposition of the common heavy atoms of the average NMR structure of (*R*)-[18-OH]ascomycin bound to FKBP to the average NMR structure of (*S*)-[18-OH]ascomycin (bold) bound to FKBP.

found in proteins. Although this is not critical in isotope-editing experiments, resulting in only a slight decrease in sensitivity if the delays are not properly set, it is very important when attempting to suppress the protons attached to ^{13}C-labeled nuclei in isotope-filtering experiments [15,16].

Fig. 3A depicts an isotope-filtered 2D NOE spectrum of (*S*)-[18-OH]ascomycin bound to [U-^{13}C,^{15}N]FKBP acquired using the pulse sequence shown in Fig. 2A. The artifacts due to the incomplete suppression of the proton resonances of ^{13}C-labeled FKBP, especially near the diagonal, make data interpretation very difficult. In contrast, using the isotope-filtered 2D NOE pulse sequence (Fig. 2B) recently developed in our laboratory [15], that employs two delays which can be tuned to different $^{1}J_{H,13C}$ values, superior suppression of ^{13}C-attached protons is obtained as illustrated in Fig. 3B. The reduction of artifacts allows more NOEs to be unambiguously interpreted in terms of proton–proton distance restraints for generating 3D structures of these ligands when bound to FKBP. Similar improvements in suppressing the proton signals of [U-^{13}C,^{15}N]FKBP were achieved in isotope-filtered 2D TOCSY spectra using doubly tuned filters (e.g. Fig. 3C), aiding in the proton resonance assignments of the bound ligands.

3.2. *Proton resonance assignments*

The proton resonances of (*R*)- and (*S*)-[18-OH]ascomycin when bound to FKBP were assigned from an analysis of isotope-filtered TOCSY spectra acquired with a mixing time of 15 ms using the pulse sequence shown in Fig. 2C. The scalar-coupled protons could readily be traced from the isotope-filtered 2D TOCSY spectra such as the one shown in Fig. 3C. The chemical shifts were assigned by a comparison with the previously assigned chemical shifts of ascomycin when bound to FKBP, and were confirmed from isotope-filtered 2D NOE data. As in ascomycin [8,9,24], protons at the 3, 4 and 5 position of the piperidine ring appear shifted to quite high field >0.3 ppm for both of the isomers. This initial observation indicated that their overall binding to FKBP must be quite similar to that of ascomycin. The overall chemical shifts for both derivatives are similar to those of ascomycin except for protons at or near the 18 position.

3.3. *Determination of the stereochemistry at the 18 position*

The oxidation of ascomycin by selenium (IV) oxide results in two diastereomers which can be separated by silica gel chromatography. Initially it was not known which stereoisomer was *R* and which was *S*. The identification of the isomers was accomplished using the NOE data. A set of 20 starting structures were generated for each isomer using distance geometry, and further refined with dynamical simulated annealing. During the structure calculations, the chirality at the 18 position was allowed to float, i.e. the chirality was allowed to flip between *R* and *S* to best statisfy the NOE constraints [25,26]. For the major isomer, all 20 structures had a stereochemistry of *S* at the 18 position after simulated annealing. For the minor isomer, 16 structures were obtained with a stereochemistry of *R*, while 4 were obtained with a stereochemistry of *S*. We concluded from this result that the major isomer was (*S*)-[18-OH]ascomycin, while the minor isomer was (*R*)-[18-OH]as-

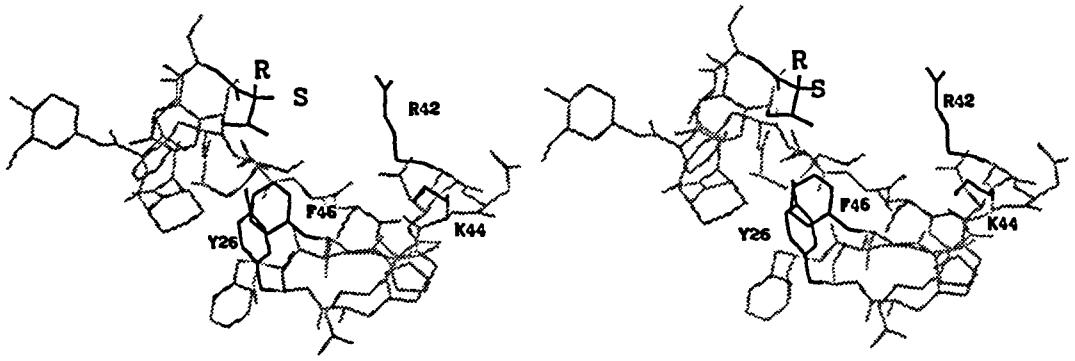


Fig. 5. Region of the ascomycin/FKBP complex [27] in the vicinity of the ascomycin binding site showing the hydrophobic cleft formed by Tyr-26, Phe-46, Arg-42, Lys-44, and part of ascomycin. The 18, pro-*R* and pro-*S* positions are labelled **R** and **S**, respectively.

comycin. Therefore, in all further structure calculations, the chirality at the 18 position was fixed.

3.4. *Bound conformation*

For the final structure calculations, 200 structures were generated for each isomer which were subsequently refined using a dynamical simulated annealing protocol. For (*S*)-[18-OH]ascomycin, 85 structures were chosen that best satisfied the NOE constraints and that had a *trans*-7,8 amide bond. The average rms deviation of all heavy atoms from a calculated average structure was 0.37 ± 0.06 Å, and no NOE violations were found greater than 0.1 Å. For (*R*)-[18-OH]ascomycin, 80 structures were chosen that best satisfied the NOE constraints. The average rms deviation of all heavy atoms from an average structure was 0.28 ± 0.11 Å, and no NOE violations were found greater than 0.1 Å. Thus, the conformations of both ligands when bound to FKBP were well defined by the NOE data. Furthermore, as indicated by the structural statistics for both analogs (Table I), the structures have relatively minor deviations from idealized geometry.

As shown in Fig. 4A and B, the bound conformations of both (*S*)- and (*R*)-[18-OH]ascomycin are very similar to the bound conformation of ascomycin. Superposition of the common heavy atoms of (*S*)-[18-OH]ascomycin and ascomycin gives an rms deviation of 0.49 Å, while superposition of the common heavy atoms of (*R*)-[18-OH]ascomycin and ascomycin gives an rms deviation of 0.36 Å. Moreover, as can be seen in Fig. 4C, the bound conformation of (*S*)- and (*R*)-[18-OH]ascomycin are virtually identical within the experimental error of the method. The rms deviation for superposition of their common heavy atoms is 0.49 Å. Thus, substitution of a hydroxyl group for a proton at the 18 positon has essentially no effect on the conformation of the bound ligand.

4. CONCLUSIONS

Using isotope-filtering techniques the proton chemical shifts of both the major and minor stereoisomers of [18-OH]ascomycin bound to FKBP were assigned. Based on distance geometry/simmulated annealing calculations, the stereochemistry at the 18 position was defined as *S* for the major isomer and as *R* for the minor isomer. In addition, from isotope-filtered 2D NOE data, the bound conformations of both isomers were precisely determined. From a comparison of the 3D structures of these analogs with that of ascomycin when bound to FKBP, it can be concluded that the lack of immunosuppressive activity displayed by these 18-hydroxy derivatives is not due to a difference in the conformation. The most likely cause of their inactivity as immunosuppressants is that the protruding 18 hydroxyl group, at either the *R* or *S* position, prevents the FKBP/ligand complex from interacting with calcineurin. An examination of the solution structure of the ascomycin/FKBP complex [27] re veals a hydrophobic cleft adjacent to bound ascomycin, which may form at least part of the calcineurin binding site. It is formed by the side chains of Tyr-26, Phe-46, and the aliphatic portion of Arg-42 and Lys-44, as well as a hydrophobic portion of ascomycin which includes the methylene group at the 18 position (Fig. 5). If this is, in fact, part of a hydrophobic, calcineurin binding site, the addition of a hydroxyl group at position 18 would disrupt the hydrophobic surface and might inhibit binding of calcineurin to the [18-OH]ascomycin/FKBP complex.

In addition to the importance of the structural information provided in these studies on FK506 analogs bound to FKBP, this work represents a good example of how isotope filtering can be used to determine the conformation of an unlabeled ligand bound to a ^{13}C-labled protein. In many cases, the synthesis of isotopically enriched drug molecules will not be possible, suggesting that this approach will be the method of choice for providing this kind of structural information to aid in the design of improved pharmaceutical agents.

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REFERENCES

[1] Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L. (1989) Nature 341, 758–760.

[2] Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. and Sigal, N.H. (1989) Nature 341, 755–757.

[3] Liu, J., Farmer Jr., J.D., Lane, W.S., Friedman, J., Weissman S.L. and Schreiber, S.L. (1991) Cell 66, 807–815.

[4] Kawai, M., Lane, B.C., Petros, A.M., Hsieh, G., Mollison, K.W., Fesik, S.W., Holzman, T.F., Carter, G.W. and Luly, J.R. (in preparation).

[5] Arai, T., Koyama, Y., Suenaga, T. and Honda, H. (1962) J. Antibiot. 15, 231–232.

[6] Hatanaka, H., Kino, T., Miyata, S., Inamura, N., Kuroka, A., Goto, T., Tanaka, H. and Okuhara, M. (1988) J. Antibiot. 41, 1592–1601.

[7] Dumont, F.J., Staruch, M.J., Koprak, S., Siekierka, J., Kindt, V., Lin, S., Harrison, R., Beattie, T. and Sigal, N.H. (1992) FASEB Meeting, April 5–9, 1992, Anaheim, CA.

[8] Petros, A.M., Gampe Jr., R.T., Gemmecker, G., Neri, P., Holzman, T.F., Edalji, R., Hochlowski, J., Jackson, M., McAlpine, J., Luly, J.R., Pilot- Matais, T., Pratt, S. and Fesik, S.W. (1991) J. Med. Chem. 34, 2925–2928.

[9] Petros, A.M., Gemmecker, G., Neri, P., Olejniczak, E.T., Nettesheim, D.G., Xu, R.X., Gubbins, E.G., Smith, H. and Fesik, S. W. (1992) J. Med. Chem. (in press).

[10] Fesik, S.W., Luly, J.R., Erickson, J.W. and Abad-Zapatero, C. (1988) Biochemistry 27, 8297–8301.

[11] Fesik, S.W., Gampe Jr., R.T., Holzman, T.F., Egan, D.A., Edalji, R., Luly, J.R., Simmer, R., Helfrich, R., Kishore, V. and Rich, D.H. (1990) Science 250, 1406–1409.

[12] Weber, C., Wider, G., von Freyberg, B., Traber, R., Braun, W., Widmer, H. and Wüthrich, K. (1991) Biochemistry 30, 6563–6574.

[13] Fesik, S.W., Gampe Jr., R.T., Eaton, H.L., Gemmecker, G., Olejniczak, E.T., Neri, P., Holzman, T.F., Egan, D.A., Edalji, R., Simmer, R., Helfrich, R., Hochlowski, J. and Jackson, M. (1991) Biochemistry 30, 6574–6583.

[14] Wider, G., Weber, C., Traber, R., Widmer, H. and Wüthrich, K. (1990) J. Am. Chem. Soc. 112, 9015–9016.

[15] Gemmecker, G., Olejniczak, E.T. and Fesik, S.W. (1992) J. Magn. Reson. 96, 199–204.

[16] Ikura, M. and Bax, A. (1992) J. Am. Chem. Soc., 114, 2433–2440.

[17] Edalji, R., Pilot-Matais, T.J., Pratt, S.D., Egan, D.A., Severin, J.M., Gubbins, E.G., Petros, A.M., Fesik, S.W., Burres, N.S. and Holzman, T.F. (1992) J. Prot. Chem. (in press).

[18] Otting, G., Senn, H., Wagner, G. and Wüthrich, K. (1986) J. Magn. Reson. 70, 500–505.

[19] Wüthrich, K., Billeter, M. and Braun, W. (1983) J. Mol. Biol. 169, 949–961.

[20] Nilges, M., Clore, M. and Gronenborn, A.M. (1988) FEBS Lett. 239, 129–136.

[21] Kuszewski, J., Nilges, M. and Brunger, A.T. (1992) J. Biomol. NMR 2, 33–56

[22] Brunger, A.T. XPLOR Manual, version 2.1. 1990.

[23] Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S. and Karplus, M.J. (1983) Comput. Chem. 4, 187–93.

[24] Wandless, T.J., Michnick, S.W., Rosen, M.K., Karplus, M. and Schreiber, S.L. (1991) J. Am. Chem. Soc. 113, 2339–2341.

[25] Weber, P.L., Morrison, R. and Hare, D.J. (1988) J. Mol. Biol. 204, 483–487.

[26] Holak, T.A., Nilges, M. and Oschkinat, H. (1989) FEBS Lett. 242, 218–224.

[27] Meadows, R.P., Xu, R.X., Nettesheim, D., Olejniczak, E.T., Petros, A.M., Holzman, T.F., Severin, J., Gubbins, E., Smith, H. and Fesik, S.W. (submitted for publication)