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Determination of heteronuclear long-range couplings to heteronuclei in natural abundance by two- and three-dimensional NMR spectroscopy

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Dedicated to the memory of Professor V.F. Bystrov

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SUMMARY

A method to determine heteronuclear long-range couplings to carbon and nitrogen at natural abundance is presented and applied to two cyclic hexapeptides and the peptidomacrolide FK506. The method is applicable for proton-bearing heteronuclei. By introduction of heteronuclear half-filters in two- or three-dimensional experiments the spectra exhibit an E.COSY pattern when executed without heteronuclear decoupling. The extraction of the heteronuclear coupling constants is therefore independent of linewidth.

In *cyclo*(-Ala-Ala-Ala-Pro-Ala-Pro-) a ^{13}C-ω_1-half-filtered TOCSY spectrum yields the $^3J(H^N\text{-}C^\beta)$ coupling constant, which can be used to remove ambiguity in the φ angle determination from $^3J(H^N\text{-}H^\alpha)$. In *cyclo*(-D-Pro-Phe-Phe-Lys(Z)-Trp-Phe-) a ^{15}N-ω_1-half-filtered TOCSY was applied to individually assign the diastereotopic β-methylene protons via the $^3J(H^\beta\text{-}N)$. In FK506 a 3D-HMQC-TOCSY without heteronuclear decoupling is used to obtain a number of heteronuclear coupling constants to carbons. These values have been applied for the assignment of diastereotopic methylene protons and determination of dihedral angles in the cyclic portion of the molecule.

INTRODUCTION

NMR spectroscopy has become a standard method for the determination of structure and dynamics of polypeptides, proteins and other biologically important compounds in solution (Wüthrich, 1986; Kaptein et al., 1988). For the most part structurally relevant parameters are obtained from homonuclear ^1H NMR-experiments as COSY, TOCSY, NOESY and ROESY (Ernst et al., 1987; Kessler et al., 1988a) and then applied to derive structures in solution. So far, hete-

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ronuclear experiments are mainly used to resolve overlap of proton signals due to the much higher chemical shift dispersion in the dimension of the heteronucleus (Fesik and Zuiderweg, 1990; Kay et al., 1990). The application of homonuclear and heteronuclear coupling constants to obtain structural information via Karplus-type relations (Karplus, 1959,1963) in peptides has been pioneered by V. Bystrov and others (Bystrov, 1976; Gavrilov et al., 1976). At that time, however, suitable techniques to determine heteronuclear coupling constants in larger molecules with the heteronucleus in natural abundance were not available.

Thus the structures derived today from NMR are mainly based on distances between protons, that are indirectly extracted from NOESY spectra (Wüthrich, 1986; Neuhaus and Williamson, 1989). However, exclusive use of NOE constraints in structure determination has its disadvantages. Spectra of globular proteins that are usually rather compact may provide enough NOE information to yield reliable structures. On the other hand, if insufficient NOE information is available, e.g. in less dense structures or on the surface of molecules, the structures are less well defined. This is generally true for smaller molecules where the surface-to-core ratio is larger than for large molecules. Another problem is the often missing stereospecific assignment of methylene protons or isopropyl methyl groups. This usually forces one to use a pseudo-atom approach (Wüthrich et al., 1983), which decreases the informational content of the NOE constraints, since the range of possible distances is increased by the distance between the proton and the pseudo-atom. Computational approaches to the latter problem have recently become popular and might prove useful in the future (Weber et al., 1988; Güntert et al., 1989; Nilges et al., 1990).

Both of these problems can be dealt with by the use of coupling constants, either homonuclear or heteronuclear, since they provide complementary structural information to the NOE constraints. In addition, the information from the coupling constants can be used for stereospecific assignments by an analysis of the side-chain population based on both types of coupling constants (Kessler et al., 1987). In some cases homonuclear coupling constants in combination with NOE intensities can be used to establish a stereospecific assignment (Kessler et al., 1984; Zuiderweg et al., 1985; Wagner et al., 1987; Kim and Prestegard, 1990), but in contrast to the combined use of heteronuclear and homonuclear coupling constants, a general procedure has not been developed.

The extraction of accurate coupling constants from NMR spectra is often difficult, since the coupling constants of interest may be of similar size or even smaller than the linewidth. This is especially true for macromolecules with short T_2 times. The values extracted from such spectra are deceiving and their use as structural constraints is diminished (Neuhaus et al., 1984). In the case of heteronuclear coupling constants, an additional problem is the low natural abundance of the heteronucleus that makes the determination of the coupling constants even more difficult. The demand for sensitive methods to determine coupling constants independent of linewidth is especially important with natural products, where the availability or solubility of the material prevents a higher concentration and isotopic labeling is not possible or prohibitively expensive. There is, however, a difference in the required accuracy, depending on what one wants to obtain from the extracted coupling constants. If the goal is only a stereospecific assignment, then a qualitative measurement may be sufficient, and the information that a coupling constant is 'large' or 'small' usually makes the desired assignment possible. To obtain structural information by transforming the coupling constant into a certain range of dihedral angles via a Karplus-Bystrov-type equation, the highest possible accuracy is required.

Here we present the determination of heteronuclear long-range couplings of three molecules,

the model peptide *cyclo*(-Ala-Ala-Ala-Pro-Ala-Pro-) (**1**), the cyclic inhibitor of a hepatic transport system *cyclo*(-D-Pro-Phe-Phe-Lys(Z)-Trp-Phe-) (**2**) and the potent immunosuppressive drug FK506 (**3**) (Kino et al., 1987a,b; Tanaka et al., 1987). The extracted heteronuclear coupling constants either allow a stereospecific assignment of diastereotopic groups or are of structural importance.

MATERIALS AND METHODS

The hexapeptides **1** and **2** have been synthesized from their linear precursors H-(Xxx)$_6$-NHNH$_2$ via the azide method (Klausner and Bodanszky, 1974). The linear precursors were prepared by the solid-phase method as described previously (Kessler and Eiermann, 1982; Kessler et al., 1982). 10 mg of **1** and 20 mg of **2** were dissolved in 0.5 ml DMSO-d_6; all measurements were done at 300 K. FK506 (**3**) was obtained from Fujisawa Pharmaceutical Company and used without further purification. 25 mg were dissolved in 0.5 ml $CDCl_3$; all measurements were done at 300 K.

NMR spectra were recorded on Bruker AMX 500 and AMX 600 spectrometers. A broadband inverse probe was used for all experiments and the sample was not spun. The pulse sequence of the ω_1-filtered-TOCSY without heteronuclear decoupling (HETLOC, Kurz et al., 1991) and 3D-HMQC-TOCSY (Fesik and Zuiderweg, 1990) without heteronuclear decoupling are shown in Fig. 1. The phase cycle was a combination of the double difference phase cycle (Cavanagh and Keeler, 1988) and schemes for suppression of axial peaks. All spectra were recorded with a BIRD pulse (Garbow et al., 1982) prior to the actual sequence to suppress protons not bound to the heteronucleus and allow for a rapid pulsing with two scans per second (Bax and Subramanian, 1986).

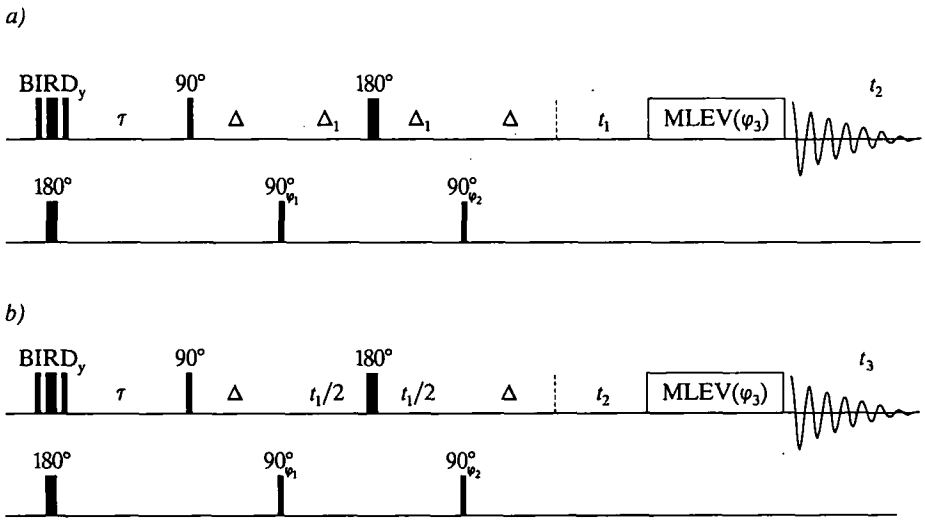

Fig. 1. Pulse sequences of the techniques applied here. (a) ω_1-filtered TOCSY without heteronuclear decoupling (HETLOC, Kurz et al., 1991), the delays are $\Delta = (2*J_{HX})^{-1}$, $\Delta_1 = 5$ μs. The delay τ has to be optimized according to the relaxation time of the molecule (Bax and Subramanian, 1986). (b) 3D-HMQC-TOCSY without heteronuclear decoupling, Δ and τ are the same as in (a). Phase cycling: $\varphi_1 = x, -x, x, -x$; $\varphi_2 = x, x, -x, -x$; $\varphi_3 = y, y, y, y, -y, -y, -y, -y$; rec = +, −, −, +. Quadrature detection in the indirect detected dimensions is achieved by TPPI (Marion and Wüthrich, 1983), applied to all phases in front of the relevant evolution time.

The ^{13}C-HETLOC (Kurz et al., 1991) of **1** was recorded at 600 MHz. The size in F_2 was 2048 points with 512 experiments of 128 scans each (total measuring time 9 h). The spectral width was 7120 Hz in F_2 and F_1. The carrier was positioned at 4.65 and 39.0 ppm for protons and carbons, respectively.

The ^{15}N-HETLOC (Kurz et al., 1991) of **2** was recorded at 500 MHz. The size in F_2 was 2048 points with 200 experiments of 384 scans each (total measuring time 14 h). The spectral width was 6250 Hz in F_2 and 2300 Hz in F_1. In F_1 the H^N protons were folded from outside the spectral window into the recorded region to optimize the resolution in the indirect detected dimension. The carrier was positioned at 5.4 ppm for protons and in the center of the nitrogen region. An HMQC (Müller, 1979; Bax et al., 1983; Bendall et al., 1983) was recorded to check the carrier position (measuring time 35 min, 16 scans, 64 experiments, data not shown).

The 3D-HMQC-TOCSY of **3** was recorded at 500 MHz. The size in F_3 was 2048 points, the number of experiments was 7808 [64 in the carbon dimension (F_1), 122 in the proton dimension (F_2)] with 64 scans each, resulting in a total measuring time of 70 h. The spectral width was 4000 Hz in F_3, 3012 Hz in F_2 and 3289 Hz in F_1. In the ^{13}C-dimension the resonances were folded after careful inspection of the HMQC. To allow for simultaneous phasing of all signals the first value for t_1 was set in a way that the linear phase correction necessary was exactly 360 degrees (Schmieder et al., 1991a). The carrier was positioned at 3.50 and 50.0 ppm for protons and carbons, respectively. Processing of the data was done on Bruker X32 data stations in case of the two-dimensional spectra. The three-dimensional spectrum was processed on a Silicon Graphics 4D/240SX computer with the software of Dr. D. Hare (FELIX). After the transformation slices were read out of the three-dimensional matrix with some simple homewritten programs in C and transferred to the X32 station for plotting.

Discussion of the methods

Since heteronuclear coupling constants are structurally important, several methods for their extraction have been proposed in the literature. A general solution, however, has not been found and will not be presented here. The applicability of the different methods depends on the type of spectroscopic problem one wishes to solve. We want to focus on methods suitable for biologically important compounds of moderate size, that are normally available only in small amounts, slightly soluble and have the heteronucleus in natural abundance. It is obvious that the method with the highest sensitivity and accuracy will be the method of choice. Therefore the execution of proton-detected experiments is a must, which excludes those methods proposed in the literature that utilize detection of the heteronucleus (Bax and Freeman, 1982; Bax, 1983; Davis et al., 1983; Jippo et al., 1986; Krishnamurthy and Casida, 1989; Uhrín and Liptaj, 1989; Uhrín et al., 1989; Waterhouse, 1989). In addition, the independence of the measurement from linewidth and high resolution in the dimension in which the heteronuclear coupling constant is measured are important factors, again excluding some of the methods proposed in the literature (Kessler et al., 1988b; Pratum et al., 1988). Some of the methods with proton detection proposed in the literature utilize selective or semiselective heteronuclear pulses. This can be of interest if only a few coupling constants are important, but generally the procedure does not differ from the nonselective experiment (Ochs and Berger, 1990; Crouch and Martin, 1991).

One important concept to determine coupling constants independent from the linewidth is the creation of E.COSY-type patterns, from which the coupling constant is extracted by measuring

the displacement of basic patterns. If homonuclear coupling constants are to be determined, the original E.COSY procedure (Griesinger et.al., 1985,1986,1987) works with a suitable combination of COSY spectra with multiple quantum filters of different order to select either connected or non-connected transitions.

Recently, Wagner and coworkers (Montelione et al., 1989) have demonstrated that heteronuclear coupling constants to a proton-bearing heteronucleus can be measured accurately from homonuclear spectra (either TOCSY or NOESY) of isotopically enriched proteins. This method relies on the fact that the heteronucleus, which is not pulsed during a homonuclear experiment, does not change its spin state and only connected transitions appear in the spectrum. The resulting correlation between the proton bound to the heteronucleus and the remote proton exhibits an E.COSY-type pattern, in which the desired heteronuclear long-range coupling is visible as a displacement of two peaks, that are separated by a large heteronuclear one-bond coupling. Thus the desired coupling constant can be measured with the usual high intensity of a homonuclear spectrum and is independent of lineshape. Here we show that in the case of smaller molecules the same effect can be achieved by implementing a proper half-filter (Otting et al., 1986) in the homonuclear technique. An extension of this sequence to a three-dimensional experiment is then straightforward (Wider et al., 1989; Edison et al., 1991). In the case of a protein, the overlap of resonances will make this extension to a higher dimension necessary. This principle has also recently been applied to the measurement of the important homonuclear $^3J(H^N\text{-}H^\alpha)$ coupling constants using a triple resonance experiment. There the large coupling constant is the $^1J(H^\alpha\text{-}C^\alpha)$ and the E.COSY pattern is created by application of a TANGO pulse (Wimperis and Freeman, 1984), effective only for the H^N protons (Montelione and Wagner, 1989,1990; Wagner et al., 1991; Schmieder et al., 1991b).

However, there are limitations to this method. The proton bound directly to the heteronucleus is of crucial importance, since this provides the large coupling constant needed to make the measurement independent of linewidth and to make an efficient transfer between proton and heteronucleus possible, thus providing the high sensitivity. If the molecule under investigation is a polypeptide, then information on the coupling constants between protons and carbonyl carbons $[^3J(H^N_{(i)}\text{-}C'_{(i)})$; $^3J(H^\alpha_{(i)}\text{-}C'_{(i+1)})]$ is structurally important. Obviously, this can not be obtained following the methodology described above.

Another drawback, only of interest if the heteronucleus is in natural abundance, is the sensitivity which is greatly reduced by the low natural abundance of the heteronucleus. This is, of course, especially severe with nitrogen as the heteronucleus. However, we will show that coupling constants between the H^β-protons and the nitrogen of the same amino acid can be obtained by a HETLOC with ^{15}N as the heteronucleus in natural abundance, providing the information necessary for stereospecific assignments. But the utilization of a through-space transfer by NOE or ROE to obtain the important coupling constant via the peptide bond $[^3J(H^\alpha_{(i)}\text{-}N_{(i+1)})]$ might lead to unacceptably long measuring times.

The only way to obtain information on the coupling to the heteronucleus that is not directly connected to a proton is the utilization of a heteronuclear long-range correlation. Unfortunately this experiment is rather insensitive, even in the proton-detected version, the HMBC (Bax and Summers, 1986). In addition, there is no reasonable way to extend it to a further dimension in case of extensive overlap. However, several methods based on this experiment have been proposed in the literature. The method developed by Keeler and coworkers meets the demand for high accura-

cy (Keeler et al., 1988; Titman et al., 1989). This method is based on an earlier proposal by Bermel et al. (Bermel et al., 1989). If executed with a sample having the heteronucleus in natural abundance, the difference between a cross section of the HMBC in the proton dimension and a reference proton spectrum is the desired heteronuclear long-range coupling constant and a phase distortion produced by the long delay necessary to develop the long-range coupling. The latter can easily be simulated by a proton spectrum with an identical delay as in the HMBC and the coupling constant can then be determined by a fitting procedure, fitting the desired heteronuclear coupling and the intensities. In case of overlap in the proton spectrum the reference multiplet can also be obtained from an appropriate, better resolved two-dimensional spectrum (e.g. a z-filtered TOCSY (Rance, 1987; Shaka et al., 1988; Titman et al., 1989)). The same method is also applicable for the determination of homonuclear coupling constants using a COSY multiplet and a reference multiplet as described above (Titman and Keeler, 1990). It is then an extension of the DISCO procedure (Kessler et al., 1985).

This method for heteronuclear coupling constants can be applied to carbon and nitrogen, but one has to keep in mind, that long measuring times might be necessary to obtain an HMBC with sufficient quality and resolution, given a sample of low concentration. Then the relatively long measuring times for the ω_1-filtered spectra with through-space transfer might be advantageous because of the relative ease with which the coupling constants can be extracted. However, to obtain coupling constants to the carbonyl carbon, the HMBC is the only possible method. Unfortunately the correlations between the carbonyl carbons and protons three bonds away are often extremely weak in HMBC spectra of polypeptides.

RESULTS AND DISCUSSION

All of the coupling constants of interest in the three molecules discussed below involve heteronuclei bearing protons. Thus only a modification of the method of Wagner et al. (Montelione et al., 1989; Kurz et al., 1991) was applied, since it is the most sensitive and easily applicable method with proton-bearing heteronuclei.

Cyclo(-Ala-Ala-Ala-Pro-Ala-Pro-), **1**

The cyclic hexapeptide **1** is one of a series of model peptides currently synthesized and investigated in our laboratory (M. Kurz, H. Kessler, unpublished results). The rather small number of protons and thus the rather small number of NOE constraints make additional structural information highly desirable. To get more information on the conformation of the backbone of the cyclic hexapeptide, we determined the heteronuclear three-bond coupling constants from the H^N to C^β, that yield information about the φ angle complementary to the $^3J(H^N\text{-}H^\alpha)$ homonuclear coupling constant.

The use of one heteronuclear coupling constant alone to give structural information by transforming it into a dihedral angle via a Karplus-Bystrov-type equation certainly has many pitfalls. The reason is the lack of reference material to obtain a good calibration of Karplus-Bystrov-type equations and the resulting uncertainty inherent of the transformation from coupling constant to dihedral angle. In addition the step from the coupling constant to the dihedral angle usually is ambiguous, producing up to four different angles. On the other hand, the utilization of more than one coupling constant, e.g. both the $^3J(H^N\text{-}H^\alpha)$ and $^3J(H^N\text{-}C^\beta)$, will allow for exclusion of certain

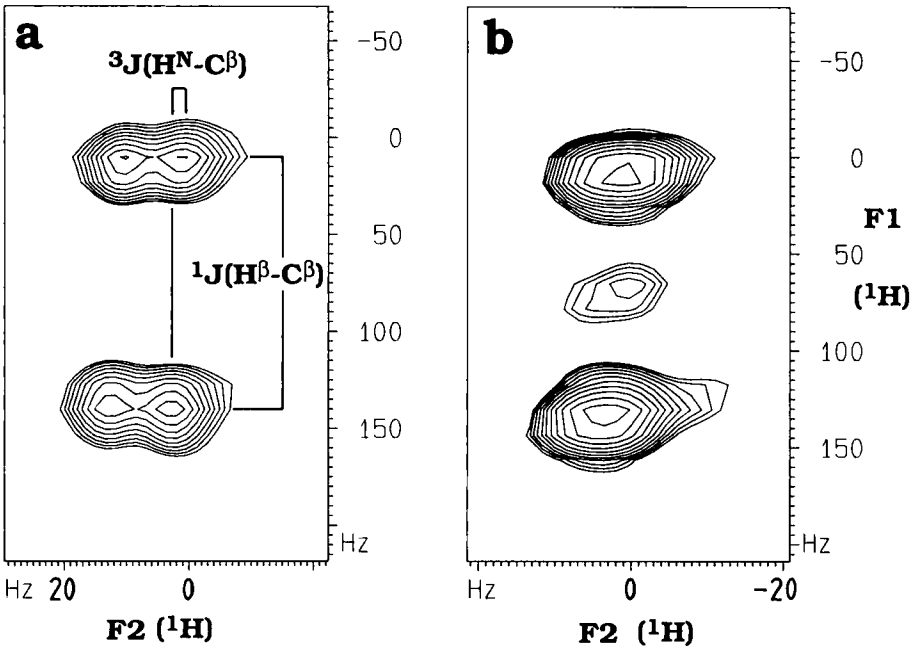

Fig. 2. Regions from the ^{13}C-HETLOC of **1**, the H^N-H^β correlations of Ala^5 (a) and Ala^1 (b) are shown. The coupling constant can easily be deduced from the displacement of the two peaks. In the center of the peak in (b) the residual signal from the protons bound to ^{12}C is visible.

possibilities even if rather large ranges of dihedral angles are given (Bystrov et al., 1977). However, it is possible that ambiguity still remains after utilizing the two coupling constants. Then two other coupling constants defining the φ-angle can help. The $^3J(H^N\text{-}C'_{(i)})$ and the $^3J(H^\alpha\text{-}C'_{(i-1)})$ can be qualitatively estimated from the long-range correlation, usually a selective HMBC (Bermel et al., 1989; Kessler et al., 1990a). It can then be compared with the value from the Karplus-Bystrov equation to resolve the ambiguity. Often the absence or presence of a cross peak will be a sufficient criterion for a discrimination of the two possibilities.

The determination of the desired coupling constants proved to be quite straightforward with **1**. A ^{13}C-HETLOC yielded correlations between the H^β bound to ^{13}C and the H^N. The heteronuclear coupling constants are determined by measuring the displacement of the two peak components as demonstrated in Fig. 2 and are given in Table 1 together with the homonuclear $^3J(H^N\text{-}H^\alpha)$ coupling constant that has been extracted from the one-dimensional proton spectrum. Since the homonuclear $^3J(H^N\text{-}H^\alpha)$ coupling constant of Ala^3 is very small, no TOCSY peak is visible after a mixing time of 80 ms and thus the determination of the heteronuclear coupling constant is not possible. Table 2 gives a comparison of the allowed dihedral angles deduced from Karplus-Bystrov-type equations (Bystrov, 1976) and the coupling constants from Table 1. The following equations (plotted in Fig. 3) have been used:

$$^3J(H^N\text{-}H^\alpha) = 9.4*\cos^2(\varphi - 60) - 1.1*\cos(\varphi - 60) + 0.4$$
$$^3J(H^N\text{-}C^\beta) = 4.5*\cos^2(\varphi + 60) - 1.5*\cos(\varphi + 60) - 0.2$$
$$^3J(H^N\text{-}C'_{(i)}) = 5.7*\cos^2(\varphi) - 2.7*\cos(\varphi) + 0.1$$
$$^3J(H^\alpha\text{-}C'_{(i-1)}) = 9.0*\cos^2(\varphi + 120) - 4.4*\cos(\varphi + 120) - 0.8$$

TABLE 1
HOMONUCLEAR $^3J(H^N\text{-}H^\alpha)$ AND ALL HETERONUCLEAR TWO- AND THREE-BOND COUPLING CONSTANTS (IN Hz) OF **1** EXTRACTED FROM THE ONE-DIMENSIONAL ^{1}H-SPECTRUM AND ^{13}C-HETLOC, RESPECTIVELY

	$^3J(H^N\text{-}H^\alpha)$	$^3J(H^N\text{-}C^\beta)$	$^2J(H^N\text{-}C^\alpha)$	$^2J(H^\alpha\text{-}C^\beta)$	$^2J(H^\beta\text{-}C^\alpha)$
Ala^1	7.1	2.4	2.4	−4.8	−4.5
Ala^2	4.8	0.4	2.5	−3.1	−4.1
Ala^3	< 1[a]	–[b]	–[b]	−4.0	−3.7
Ala^5	7.9	1.6	1.7	−5.5	−4.0

[a] Coupling constant could not be extracted from the one-dimensional spectrum.
[b] Coupling constant could not be determined, since no correlation was visible in the HETLOC spectrum.

where φ is the angle between both carbonyl carbons (according to the IUPAC nomenclature rules). From the first two equations numerical values can be extracted, that have to be understood as the center of an allowed region. Remaining ambiguities are then resolved by calculating the intensity of a correlation in the HMBC from the coupling constant using the other two equations and comparing the expected intensity with that found in the spectrum. Those four coupling constants together are usually sufficient to exclude all regions of φ-angles except one. However, in Ala^1 overlap between the carbonyl carbon of Ala^1 and Pro^6 (data not shown) prevented a differentiation between $\varphi = -82$ and $\varphi = 76$ (see Table 2).

Beside the three-bond couplings, the two-bond couplings also deserve comment. The two-bond $^2J(H^\beta\text{-}C^\alpha)$ is a coupling constant from methyl protons to a carbon. For symmetry reasons, the three staggered rotamers are equally populated and only one average coupling constant occurs for all amino acids. A comparison of these coupling constants thus gives a hint of the accuracy of the values obtained in this particular situation. The agreement is acceptable, since the uncertainty

TABLE 2
THE φ-ANGLES OF THREE ALANINE RESIDUES OF **1**, DERIVED FROM THE COUPLING CONSTANTS GIVEN IN TABLE 1 VIA THE KARPLUS-BYSTROV-TYPE EQUATIONS GIVEN IN THE TEXT

	Angles from $^3J(H^N\text{-}H^\alpha)$	Angles from $^3J(H^N\text{-}C^\beta)$	Resulting angle[a]	Angle from MD[d]
Ala^1	*85*, 35, *−82*, −158	−37, *−83*, *67*, 173	−82.5/76[b]	−75
Ala^2	102, 18, −69, *−171*	−3, −127, 43, *−163*	−167[c]	−162
Ala^5	78, 42, *−87*, −153	−23, *−97*, 59, −179	−92[c]	−63

[a] The given angles are the *mathematical* (arithmetic) average of the two most similar values from the coupling constant. Obviously they should only be considered as the center of an allowed region.
[b] No differentiation between these two values is possible, because of the symmetry of the dependence of $^3J(H^N\text{-}C'_{(i)})$ on the φ-angle and the overlap of C′ of Ala^1 and Pro^6.
[c] These values have been determined by using the information on the $^3J(H^N\text{-}C'_{(i)})$ and $^3J(H^\alpha\text{-}C'_{(i-1)})$ from the HMBC.
[d] The result of an MD calculation utilized only the NOE-constraints. The φ-angle is the mean over a 100-ps restrained MD trajectory.

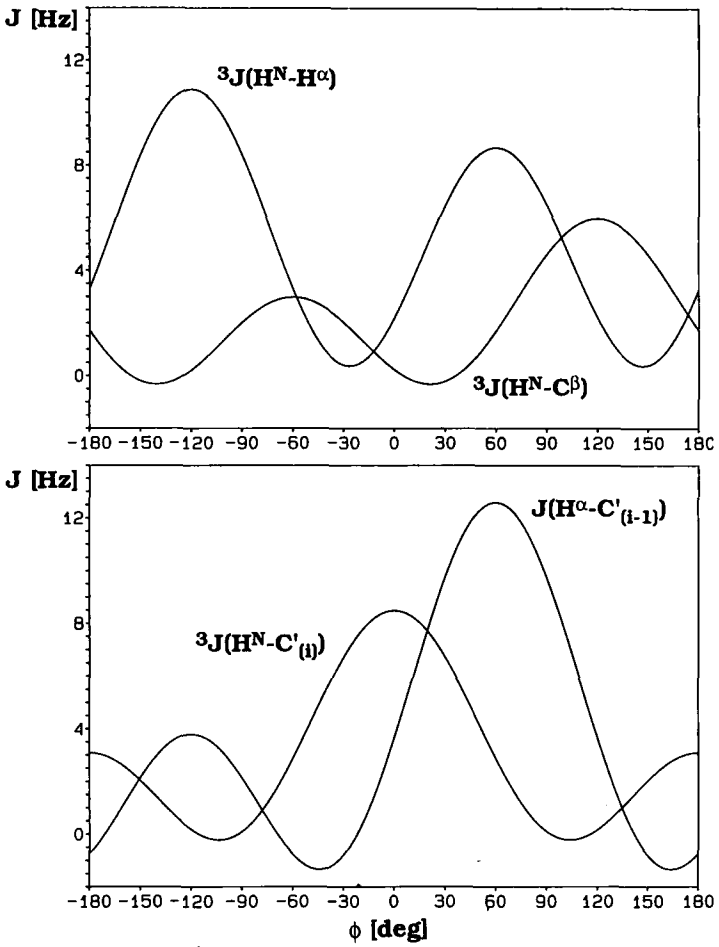

Fig. 3. Graphical representation of the Karplus-Bystrov-type equations given in the text, relevant for $^3J(H^N\text{-}H^\alpha)$, $^3J(H^N\text{-}C^\beta)$, $^3J(H^N\text{-}C'_{(i)})$ and $^3J(H^\alpha\text{-}C'_{(i-1)})$ (Bystrov, 1976). The lines represent the center of an allowed region.

seems to be only 0.5 Hz (Table 2) and the Karplus-Bystrov-type equation is normally rather steep: this will not lead to large errors in the dihedral angle.

Another two-bond coupling, the $^2J(H^N\text{-}C^\alpha)$, shows an unexpected result. The absolute sign of the coupling constant can not be determined from this experiment, but a relative sign is directly visible from the tilt of the peaks. Since it is very likely that the $^3J(H^N\text{-}C^\beta)$ is positive, the signs of the other couplings can be determined. Contrary to the literature (Bystrov, 1976), the two-bond coupling from the H^N to C^α exhibits the same sign as the three-bond coupling, while the other two observable two-bond couplings are negative, in agreement with the literature. However, the values of the $^2J(H^\alpha\text{-}C^\beta)$ are rather small, compared with the values given in the literature.

Cyclo(-D-Pro-Phe-Phe-Lys(Z)-Trp-Phe-), **2**

This cyclic hexapeptide was derived from the potent inhibitor of a hepatic transport system, '008' (Kessler et al., 1989). Only Thr^3 has been substituted by a phenylalanine (Schudok, 1989).

Stereospecific assignment of the diastereotopic H^{β} protons of the aromatic residues was required to improve the quality of the data set used for constrained MD calculations. The homonuclear coupling constants $^{3}J(H^{\alpha}\text{-}H^{\beta})$ have been extracted from an E.COSY spectrum (U. Anders, 1989, data not shown). The heteronuclear coupling constants from the H^{β} to the nitrogen were determined from an HETLOC with ^{15}N as the heteronucleus.

To be able to place the proton frequency in the center of the spectrum for an efficient TOCSY-transfer and optimum resolution in the F_1-dimension, the H^N resonances were folded. This is usually done by setting the spectral window in F_1 to the size of the region of the H^N-protons. Then the protons selected by the hetero-filter will normally be completely outside of the window and will thus be folded inside without any ambiguity or loss of information. The number of t_1 increments necessary to obtain the usual resolution of a homonuclear experiment is then drastically reduced. In Fig. 4 the region containing the correlations from H^N to H^{α} and H^{β} of an ^{15}N-HETLOC of **2** is shown; the coupling constants can be measured as described above. Unfortunately the H^{β}'s of Trp^5 overlap with the water resonance, producing a t_1-ridge that obscures the peaks. The other $^{3}J(H^{\beta}\text{-}N)$ coupling constants are given in Table 3 together with the homonuclear coupling constants.

A stereospecific assignment can either be obtained by a qualitative inspection of the three possible rotamers or more elaborately by an application of the Pachler equations (Pachler, 1963,1964; Kessler et al., 1987), leading to the same result. These procedures have been described in the litera-

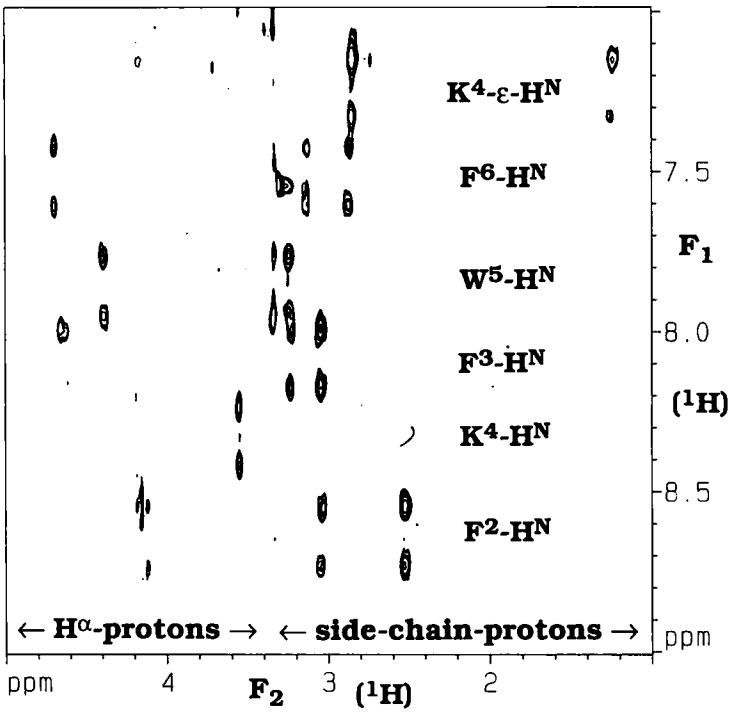

Fig. 4. ^{15}N-HETLOC of **2** with the region of the correlation from H^N to aliphatic protons shown. The opposite tilt of the signals to the H^{α} protons is visible, indicating an opposite sign of the coupling constant.

TABLE 3
HOMONUCLEAR AND HETERONUCLEAR COUPLING CONSTANTS (IN Hz) OF **2**, EXTRACTED FROM A THREE-SPIN E.COSY AND AN ^{15}N-HETLOC, RESPECTIVELY

	$^3J(H^\alpha\text{-}H^\beta)_l$[a]	$^3J(H^\alpha\text{-}H^\beta)_h$[a]	$^3J(H^\beta\text{-}N)_l$[a]	$^3J(H^\beta\text{-}N)_h$[a]	$^2J(H^a\text{-}N)$[b]
Phe^2	4.4	9.5	−4.2	−1.7	1.6
Phe^3	3.7	11.7	−3.7	−1.6	1.4
Phe^6	3.3	11.4	−1.7	−1.1	1.4

[a] l and h are the lowfield and highfield H^β protons, respectively.

[b] The two-bond coupling constant of Trp^5 is 2.1 Hz, the sign of the two-bond coupling constants is opposite to those of the three-bond couplings.

ture; however, with one exception (Kessler et al., 1987), up to now only compounds labeled with ^{15}N have been used (Lichter and Roberts, 1970; Sogn et al., 1973; Bystrov et al., 1977; Fischman et al., 1980; Cowburn et al., 1983; Stimson et al., 1986). The application of the Pachler equations can directly give information on the relative side-chain population.

The basic assumption is that only the three staggered conformations depicted in Fig. 5 are populated. The qualitative method shall be described for Phe^6. If the angle subtended by the two coupling partners is 180° the coupling constant is large; if the angle is 60°, it is small. In Phe^6 one $^3J(H^\alpha\text{-}H^\beta)$ is large, the other is small whereas both heteronuclear coupling constants are small. The latter allows only P_{II} and thus the proton with the large homonuclear coupling constant will be the pro-S proton, as can be seen in Fig. 5. Similar arguments lead to the assignment of the other methylene protons.

To apply the Pachler equations, knowledge on the coupling constants $^3J_{ap}$ (the H^β and the nitrogen are antiperiplanar) and the $^3J_{sc}$ (the H^β and the nitrogen are synclinal) is required. The values found in the literature (De Marco et al., 1978) are −5.5 and −0.4 for $^3J_{ap}$ and $^3J_{sc}$, respectively. With these values the rotamer population can be calculated from the homonuclear and heteronuclear coupling constants, in each case for both possible diastereotopic assignments of the H^β protons. Comparison of the values then gives the correct assignments as shown in Table 4.

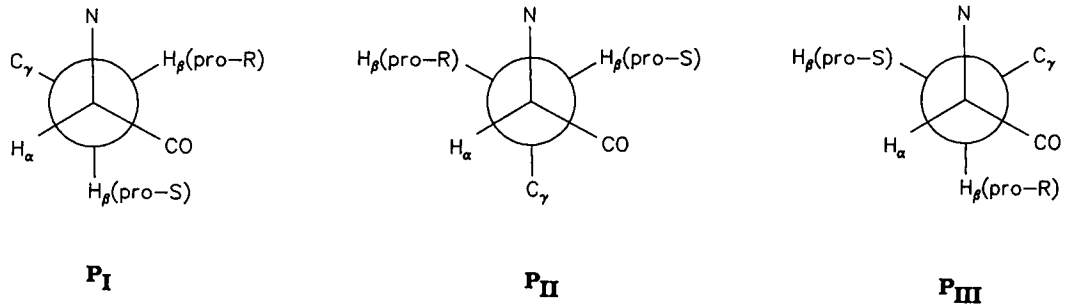

Fig. 5. Newman projection of the three staggered side-chain conformations in an amino acid with two H^β-protons. Here the χ_1 angle is relevant. The three populations are named P_I, P_{II} and P_{III} for $\chi_1 = -60$, $\chi_1 = 180$ and $\chi_1 = 60$, respectively.

TABLE 4
SIDE-CHAIN ROTAMER POPULATION AND STEREOSPECIFIC ASSIGNMENT OF THE H^{β}-METHYLENE PROTONS OF THE THREE PHENYLALANINES OF **2**

	Rotamer populations[a]						Assignment	
	P_I[b]	P_{II}[b]	P_{III}[b]	P_I[c]	P_{II}[c]	P_{III}[c]	H_l[b]	H_h[b]
Phe^2	63(16)	16(63)	21	75(25)	0	25(75)	pro-S	pro-R
Phe^3	83(10)	10(83)	7	65(23)	12	23(65)	pro-S	pro-R
Phe^6	6(80)	80(6)	14	14(25)	61	25(14)	pro-R	pro-S

[a] The values in parentheses can be excluded by a comparison of the populations from homonuclear and heteronuclear coupling constants.
[b] Populations from homonuclear coupling constants.
[c] Populations from heteronuclear coupling constants.

FK506, **3**

FK506, the structure of which is shown in Fig. 6, was isolated from a strain of *Streptomyces tsukubaensis* in 1987. It proved to be a potent immunosuppressive agent, inhibiting the early T-cell activation genes at even lower doses than necessary with Cyclosporin A, which is widely applied in organ transplantation to prevent graft rejection. The assignment of the proton and carbon NMR spectra of FK506 in $CDCl_3$ has recently been completed in our laboratory and the solution structure has been determined based on NOE information extracted from spectra recorded at 243 K. Two isomers were observed in a ratio of 2:1 and their structure could be determined separately (Karuso et al., 1990; Mierke et al., 1991). However, no stereospecific assignment was possible with the homonuclear coupling constants alone and/or NOE values for the diastereotopic protons at carbons 16, 18 and 23, that are in the important 'effector region' of FK506 (Schreiber, 1991). The

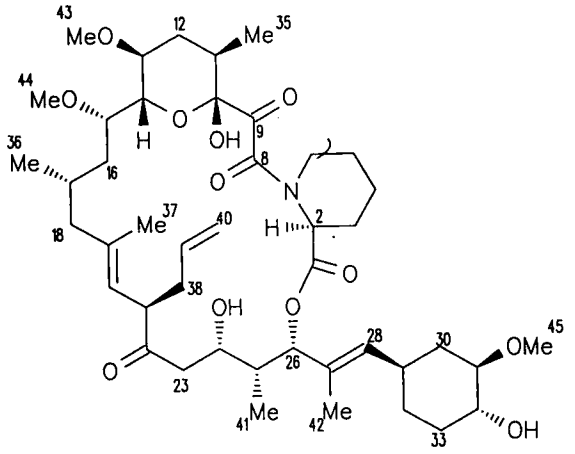

Fig. 6. Constitution of FK506, the numbering of the heavy atoms is taken from the Cambridge Data Bank.

other geminal protons could be assigned stereospecifically with homonuclear coupling constants and NOE information, since the conformation of the hexacycles is rather rigid. To obtain more structural constraints and to assign the diastereotopic protons, we determined the heteronuclear coupling constants. The similarity of the chemical shifts of both conformations required the highest resolution. A TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) of FK506

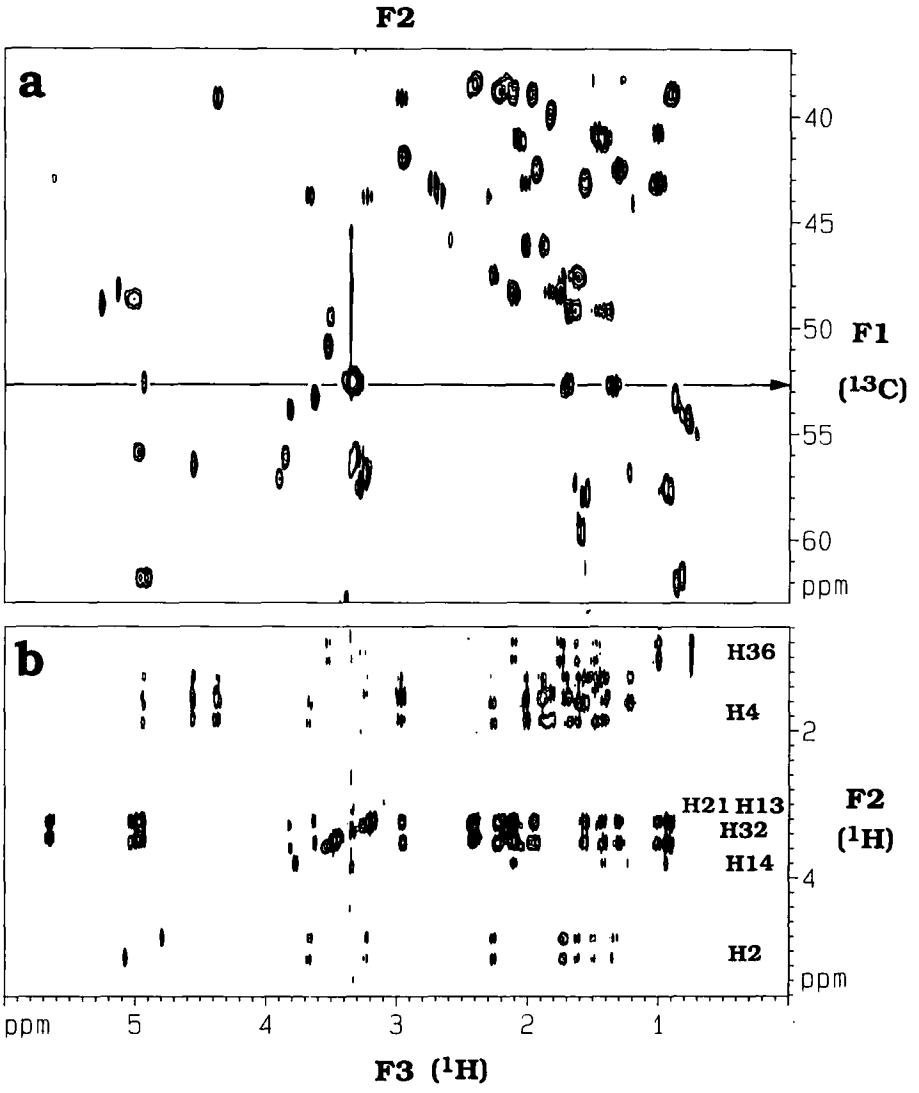

Fig. 7. Demonstration of the folding applied in the 3D-HMQC-TOCSY of 3. (a) an HMQC of 3 recorded with the same spectral width in F_1 and F_2 as in the 3D corresponding to F_1 and F_3 in the 3D, but with heteronuclear decoupling. The folding was done after careful inspection of the HMQC. In case of FK506 folding with TPPI (Marion and Wüthrich, 1983) [instead of aliasing with TPPI-States (Marion et al., 1989)] seemed to be superior. (b) F_2/F_3-slice through the 3D at 52.8 ppm on the carbon (F_1) axis. The typical patterns are visible. The folding caused almost no overlap in the spectrum. Although there is overlap of C13, C21 and C32 visible in (b) an extraction of the coupling constants is still possible.

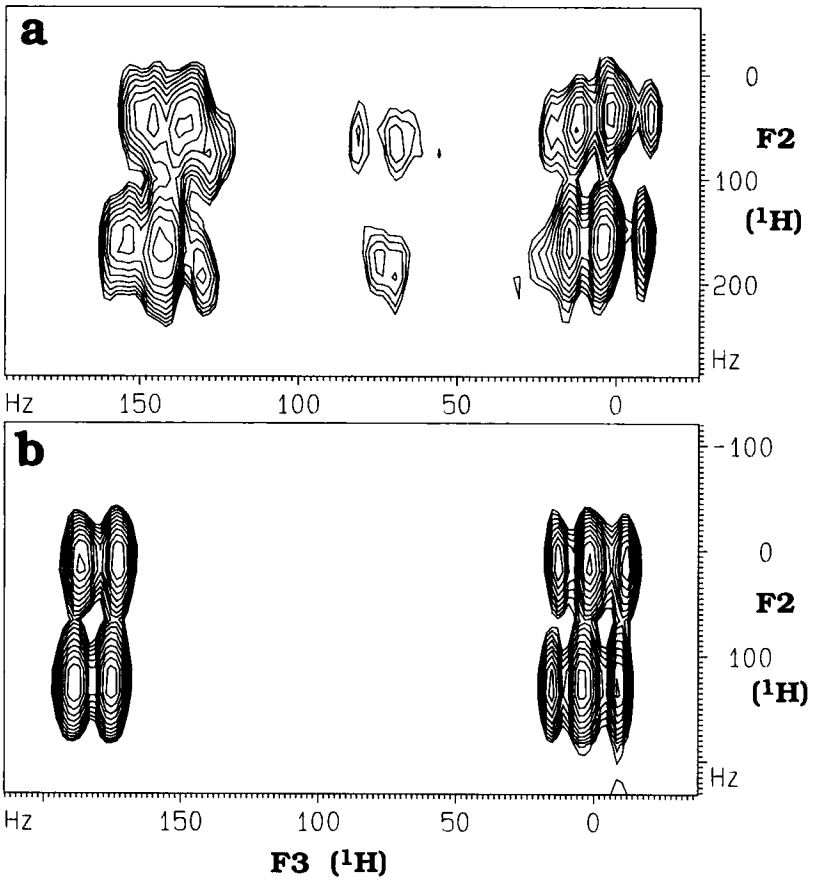

Fig. 8. Regions from the 3D-HMQC-TOCSY without heteronuclear decoupling. The correlations from H^{18} to C^{36} in the minor (a) and major (b) conformer of **3** are shown. The coupling constant that can be extracted from these signals gives the stereospecific assignment of the methylene protons of C^{18} as shown in Table 5.

(Mierke et al., 1991) immediately made it obvious that the two-dimensional approach of the method of Wagner (Montelione et al., 1989; Kurz et al., 1991), where the number of signals would be doubled, is not likely to be successful.

Neither is the method of Keeler et al. because the relatively low concentration of the sample (33 and 17 mM for the two conformers) would require unreasonably long measuring times to obtain an HMBC of sufficient quality. In addition the correlation of prime interest in the HMBC would have been from geminal protons to methyl carbons. These signals are usually weak in an HMBC. On the other hand correlations from methyl protons to geminal protons in a TOCSY are usually rather intense, making the method of Wagner et al. advantageous.

Thus we decided to record a three-dimensional heteronuclear spectrum, a 3D-HMQC-TOCSY without heterodecoupling, which, in fact, is not an 'overkill', since it has been demonstrated previously that valuable three-dimensional spectra of compounds in natural abundance can be recorded in a short time due to the extremely high sensitivity of these methods (Fesik et al., 1989;

de Waard et al., 1990; Kessler et al., 1990b; Schmieder et al., 1990). It also appeared necessary to determine not only the coupling constants in which the three methylene groups are involved, but also as many other coupling constants as possible to check the validity of the parameters in the Karplus-Bystrov-type equation, that presumably differs in a non-peptidic compound, and to get reference material from couplings of the rather rigid parts of the molecule.

The pulse sequence used is depicted in Fig. 1. It resembles the 3D-HMQC-TOCSY with a slight variation by omitting the heteronuclear decoupling schemes to allow for a determination of the heteronuclear coupling constants. The full proton spectrum was recorded in the F_2 and F_3 dimensions. To optimize the resolution the carbon spectrum was folded several times in the F_1 dimension. Since the carbons in the downfield region of the spectrum are usually bound to protons downfield in the proton spectrum no ambiguity is introduced by the folding, as can be seen in

TABLE 5

$^3J(H,H)$ and $^3J(C,H)$ COUPLING CONSTANTS OF THE MAJOR AND MINOR ISOMERS OF FK506 (3) USED FOR THE DIASTEREOTOPIC ASSIGNMENT OF METHYLENE PROTONS 16, 18 AND 23[a]

	Major	Minor
Methylene 16		
H^{16}-H^{17}	10.8	9.3
$H^{16'}$-H^{17}	5.5	–[b]
C^{36}-H^{16}	–[b]	2.8
C^{36}-$H^{16'}$	7.6	5.5
Assignment	H^{16} pro-R	H^{16} pro-R
	$H^{16'}$ pro-S	$H^{16'}$ pro-S
Methylene 18		
H^{18}-H^{17}	10.8	9.3
$H^{18'}$-H^{17}	5.5	5.3
C^{16}-H^{18}	2.5	1.7
C^{16}-$H^{18'}$	6.8	1.8
C^{36}-H^{18}	3.0	3.1
C^{36}-$H^{18'}$	3.3	7.5
Assignment	H^{18} pro-R	H^{18} pro-S
	$H^{18'}$ pro-S	$H^{18'}$ pro-R
Methylene 23		
H^{23}-H^{24}	3.4	2.3
$H^{23'}$-H^{24}	9.2	8.9
C^{25}-H^{23}	–[c]	4.7
C^{25}-$H^{23'}$	2.4	1.0
Assignment	H^{23} pro-S[d]	H^{23} pro-S
	$H^{23'}$ pro-R[d]	$H^{23'}$ pro-R

[a] Values in Hz, the prime (') indicates low-field proton.

[b] Coupling constant was not measured because of overlapping resonances.

[c] Intensity was too weak for accurate measurement of coupling constant.

[d] Assignment based on exchange peaks between the two conformations visible in the ROESY spectrum.

Fig. 7. Figure 7a shows an HMQC recorded with the same spectral width and the same number of experiments in F_1 as the three-dimensional spectrum. It is obvious that even after the extensive folding no overlap occurs. Figure 7b shows a slice of the three-dimensional spectrum. If two carbon resonances overlap, the slices still offer the advantage of two-dimensional NMR, i.e. the important correlations need not overlap as well. Figure 8 shows a region of the F_2/F_3 plane at 53.2 ppm, exhibiting the cross peak from H^{18} to C^{36}. The extraction of the coupling constant is straightforward and is done as in the two-dimensional case by measuring the displacement of the two signals separated by the large heteronuclear one-bond coupling. Following this procedure nearly all non-vanishing coupling constants could be determined, with the same accuracy as in the two-dimensional spectrum. This accuracy is sufficient to yield a stereospecific assignment of geminal protons. The stereospecific assignment can be achieved as described for the H^{β}'s in **2** and is demonstrated in Table 5. Since little experimental data are available on this kind of molecules, an extensive use of *all* heteronuclear coupling constants in the structure determination was not yet possible. However, some of the coupling constants are structurally informative (Mierke et al., 1991).

CONCLUSION

We have shown that heteronuclear long-range couplings can be conveniently extracted, independent of linewidth, in heteronuclear ω_1-filtered spectra with the heteronucleus in natural abundance. These couplings provide valuable information for the determination of three-dimensional structures based on NMR spectroscopy, leading to stereospecific assignments and ranges of possible dihedral angles.

We must point out, that the direct use of J-couplings to determine dihedral angles is based on the assumption of one rigid conformation. This is a first-order approximation. In general the observed J-value is a population-weighted mean of all conformations accessible and therefore dynamics must be considered. Within restrained MD simulations, this means that the J-value must be calculated for each structure of the trajectory and not from the dihedral angle of the mean structure. In addition, when a J-value is used to calculate a dihedral restraint, a wide range of torsions must be acceptable. However, it became obvious during this work that more reference material has to be obtained, to make the procedure more reliable.

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