75. Peptide Conformations

Part 30[1])

Assignment of the ^{1}H-, ^{13}C-, and ^{15}N-NMR Spectra of Cyclosporin A in $CDCl_3$ and C_6D_6 by a Combination of Homo- and Heteronuclear Two-Dimensional Techniques

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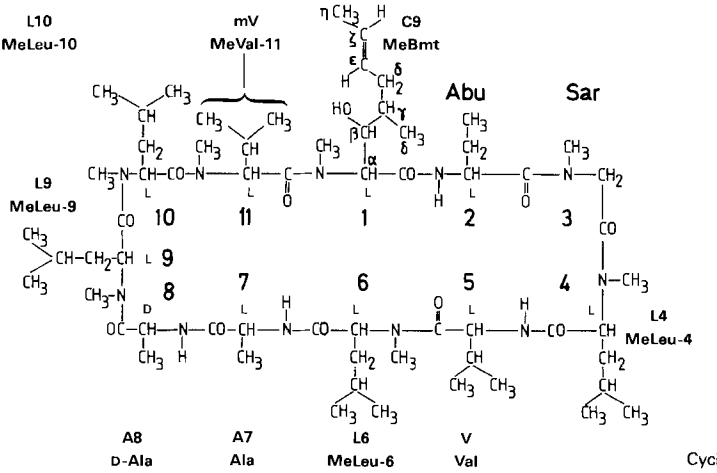
The ^{1}H-, ^{13}C-, and ^{15}N-NMR spectra of the immunosuppressive cyclic undecapeptide cyclosporin A (**1**) have been analyzed at 300 MHz in $CDCl_3$, C_6D_6, and mixtures of these solvents. A combination of different homo- and heteronuclear two-dimensional NMR techniques enable complete assignment of all H-, C- and 4 N-signals. Recognition of the proton spin systems has been achieved *via* ^{1}H,^{1}H–COSY and double-quantum-^{1}H-NMR spectroscopy. NOESY spectra yield some sequence assignments, but two techniques using coupling across amide bonds have been applied to get independent assignments of all amino acids in the sequence: (*i*) An ^{1}H,^{1}H-COSY spectrum optimized for small coupling constants enables the detection of long-range couplings from *N*-methyl groups to both α-protons attached to that amide bond. (*ii*) An ^{1}H,^{13}C-COSY spectrum optimized for C,**H**-long-range couplings (J = 5 to 10 Hz) to the eleven CO groups again yields coupling to both α-protons attached to that amide bond. Additionally these two experiments yield the assignment of *N*-methyl protons and carbonyl C-atoms. Normal and relayed ^{1}H,^{13}C-COSY in both solvents have been applied to assign all C-atoms *via* their directly attached and remote protons. An ^{1}H,^{13}C-COLOC spectrum at 500 MHz in $CDCl_3$, which uses H,C-long-range couplings confirms the assignment of all proton spin systems as well as the C-signals of each individual amino acid. Ambiguities in the assignment of the C(δ)'s of MeLeu have thus been removed. An ^{1}H,^{15}N-COSY spectrum enables the assignment of the 4 NH N-atoms.

1. Introduction. – The unique pharmacological activity of cyclosporin A (**1**) as an immuno suppressive drug [2–5] stimulated our interest in its conformation in solution [6]. NMR spectroscopy provides the most reliable experimental method for this purpose [7], but an unequivocal assignment of the NMR spectrum to molecular constitution is a prerequisite for the correct evaluation of the conformation from spectral data. We report here the total assignment of all 111 protons, all 62 C-atoms, and 4 of the 11 N-atoms of cyclosporin A in $CDCl_3$ and C_6D_6 by means of a combination of different two-dimensional NMR techniques (2D-NMR). This paper deals with the assignment of the NMR spectra whereas the subsequent communication contains the detailed discussion of the conformation in solution as well as in the crystalline state.

We want to point out that during and after the performance of this work, improved NMR techniques have been reported. For instance, phase-sensitive COSY [8] and NOESY [9] spectra in connection with improved hardware now offer higher resolution than conventional techniques. All spectra (except the H,C-COLOC) were recorded before July 1983 [10]. An account of this work has been presented at several conferences [11–13].

[1]) Part 29: [1].



Cyclosporin A (**1**)[a])

[a]) In order to facilitate the inscriptions in the *Figures*, the amino-acid residues were abbreviated either by their conventional IUPAC/IUB three-letter notations or by more arbitrary one-letter notations that are not always in accordance with the IUPAC/IUB conventions, *i.e.* MeBmt = **C9** (see [2]), MeLeu = **L**, Val = **V**, D- and L-Ala = **A**, MeVal = **mV**. Moreover, Sar (= sarcosine) means *N*-methylglycine.

Cyclosporin A (**1**) is a cyclic undecapeptide containing some interesting features: (*i*) A high content of aliphatic amino acids gives rise to strong overlap of signals in the high-field region of the spectrum. (*ii*) Seven amide N–atoms are methylated. This complicates the sequencing by the conventional procedure (combination of NOE and coupling) and requires a careful determination of *cis* or *trans* conformation about these peptide bonds. (*iii*) The occurrence of four *N*-methylated leucine residues necessitates the unravelling of overlapping complicated spin systems.

2. Assignment of ^{1}H-NMR Spectra. – 2.1. *Strategy of Assignment. In general* the analysis of peptide NMR spectra begins with the identification of the ^{1}H-NMR spin systems of the different amino acids. The following sequence assignment for those amino acids which are not directly evident from the spin system is performed *via* NOE effects from the NH proton of one amino acid to α- (or β-) protons of the preceding amino acids [14] [15] or by the comparison of the spectrum with those of specifically labeled derivatives. The assignments by NOE effects run into difficulties if corresponding signals overlap or if the NOE effects are too small for reliable interpretations.

In our case NOE effects have also been applied for the discrimination of the *cis*- and *trans*-conformations about *N*-methylated peptide bonds (CO–NCH_3). To avoid conclusions from circular arguments, we provided two independent techniques for sequence assignment by using couplings across the amide bonds: (*i*) long-range *proton* couplings from the *N*-methyl groups to both neighboring α-protons were detected *via* an optimized ^{1}H,^{1}H-COSY experiment [16]. This enabled the sequencing and assignment of the 7 *N*-methyl resonances together with their adjacent amino acids; (*ii*) heteronuclear couplings between α-protons and carbonyl C-atoms cause cross peaks in a 2D-H,C-shift correlation (^{1}H,^{13}C-COSY) optimized for smaller couplings. This experiment yields, in addition, the assignment of the carbonyl C-signals and a cross-check for the *N*-methyl H-resonances.

Ambiguities in the evaluation of the spin systems resulting from overlapping signals were removed *via* solvent titration ($CDCl_3/C_6D_6$) accompanied by COSY spectra, which allow the aromatic solvent induced shifts (ASIS) [17] of all H-resonances to be followed. Furthermore all 2D-NMR experiments presented in this paper (except the $^1H,^{13}C$-COLOC spectrum) were performed in $CDCl_3$ and C_6D_6 solution to allow a cross-check of the assignments.

It should be mentioned that the proof of the chirality of the amino-acid residues is not possible by NMR methods in a straightforward manner. In cyclosporin A, the configuration of all amino-acid residues were derived from the X-ray analysis [6] [18] and degradation studies [2]. Hence we do not focus on this fact but the conformational discussion automatically includes the configurational problem.

2.2. *Identification of Spin Systems.* The ^{1}H-NMR spectra (270 MHz) of cyclosporin A (**1**) in $CDCl_3$ and C_6D_6 are shown in *Fig. 1.* To facilitate initial interpretation of the spectra, the assignments of some resonances are given here, although experimental proof will follow below. It is evident from the spectra that one conformation strongly dominates in both solvents. Some minor peaks especially in the *N*-methyl region (2.5–3.5 ppm) are visible indicating the presence of another conformation in slow exchange. A completely different situation is observed in DMSO solution, where a mixture of at least 7 conformations leads to a spectrum of high complexity. We therefore restrict ourselves here to the lipophilic solvents. Roughly speaking, 5 more or less separated spectral regions are distinguishable: the NH protons resonate between 7 and 8.3 ppm, the α-protons as well as the olefinic protons between 4.5 and 6 ppm, the *N*-methyl groups between 2.6 and 3.8 ppm, the *C*-methyl groups between 0.6 and 1.8 ppm, and the aliphatic β-, γ-, and δ-protons in the range of 1.1–2.7 ppm.

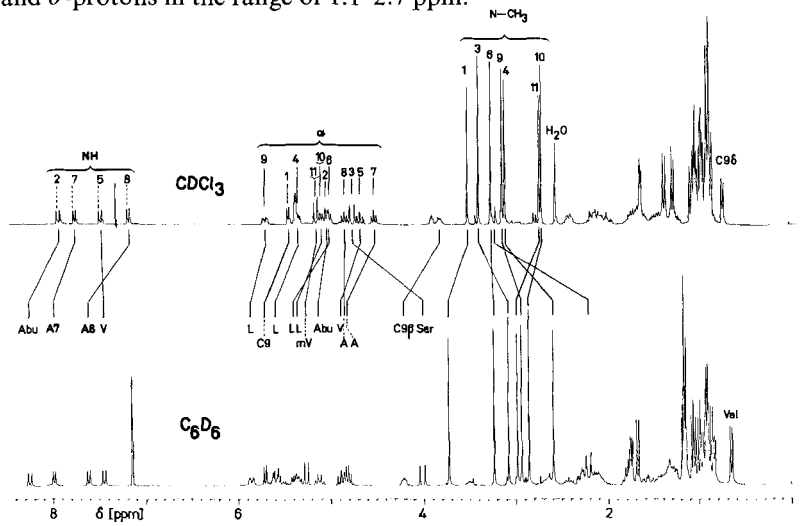


Fig. 1. *^{1}H-NMR spectra (270 MHz) of cyclosporin A (* **1***), in $CDCl_3$ (above) and C_6D_6 (below)*[2]. Some assignments are indicated by the numbers of the amino acid residues and by the one-letter symbols as shown in *Formula* **1**.

[2]) In the text dealing with ^{1}H-NMR data, the parentheses following an abbreviation of an amino-acid residue (see *Formula* **1**) contains the positional Greek letters as short form for the H-atoms bound to the corresponding C-atom. In *Figures*, these parentheses are usually omitted. Similarly in text dealing with ^{13}C-NMR data, the Greek letters refer to the C-atoms.

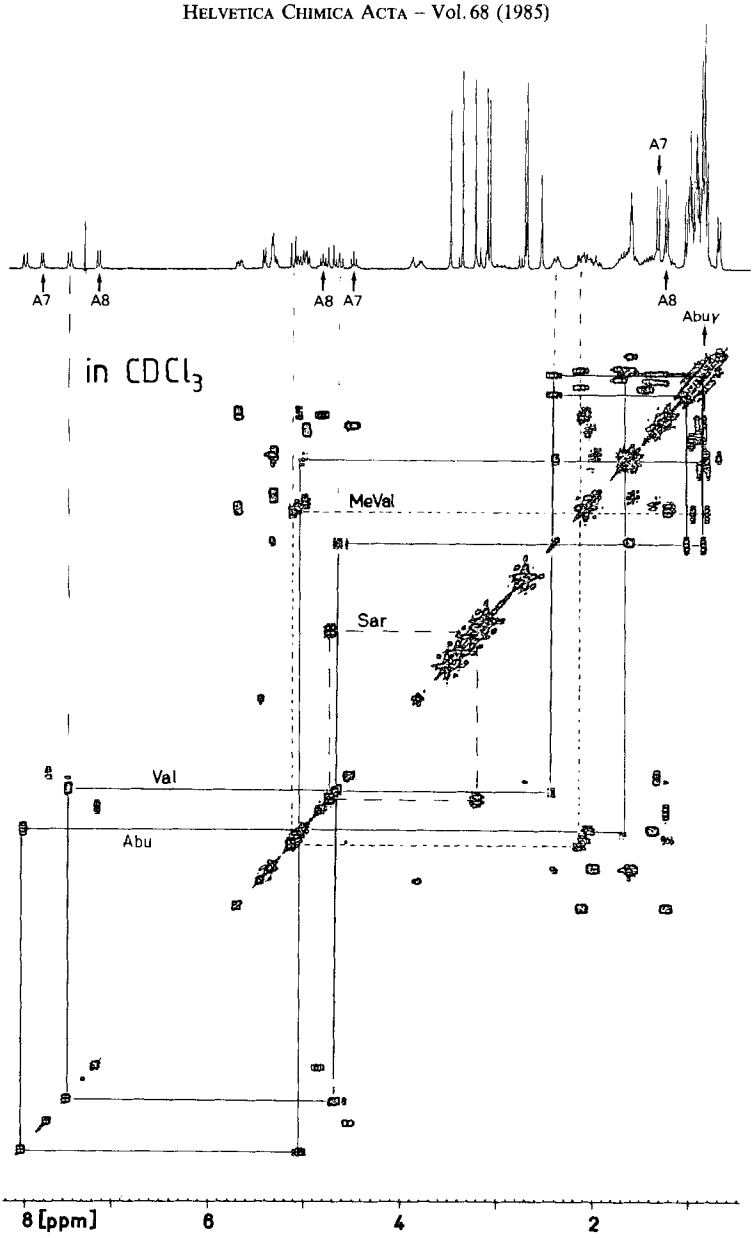


Fig. 2. *$^1H,^1H$-COSY-NMR spectrum (300 MHz) of* **1** *in $CDCl_3$*). For indications see text.

The COSY spectrum [16] [19] [20] in $CDCl_3$ *(Fig. 2)* allows a straightforward identification of spin systems of the 4 nonmethylated amino-acid residues (Val, Abu, Ala, D-Ala). Also the resonances of the MeVal and Sar residue are characterized by their typical coupling pattern *(Fig. 2)*. The remaining amino-acid residues (MeBmt [2] and 4 MeLeu's) could not be assigned from the absolute-value COSY spectrum alone: The resonances of the α- and ζ-protons as well as those of the γ- and δ-protons of MeBmt

overlap strongly. Also the β- and γ-protons of the different MeLeu residues resonate at similar chemical-shift values. The COSY spectrum in C_6D_6 *(Fig. 3)* yields a better separation of the above-mentioned signals. We have drawn lines to indicate the cross peaks of the MeBmt resonances in *Fig. 3*. All expected cross peaks except those of the β,γ coupling are visible in this plot. To detect the latter, a lower plotting level is required because the broadness of the resonances distributes the intensity over a broader spectral range.

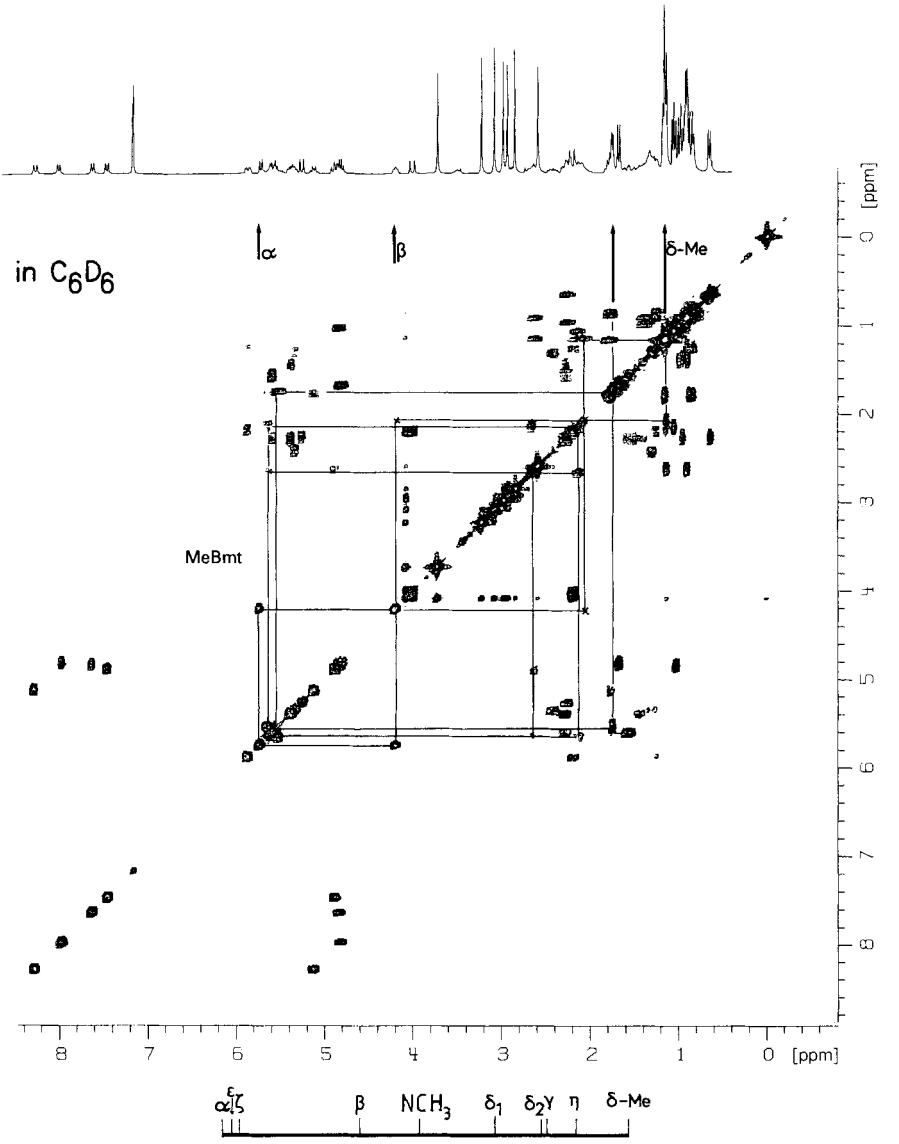


Fig. 3. *$^1H,^1H$-COSY-NMR spectrum (300 MHz) of* **1** *in C_6D_6*[2]). The spin system of MeBmt is indicated in the spectrum and at the bottom. The cross peak of the β,γ coupling is only visible at a lower-level plot; it is indicated by a cross.

Difficulties arise for the identification of the MeLeu residues: the cross peaks for the α,β couplings (2 for each MeLeu residue) as well as those of the γ-protons to both diastereotopic methyl groups were clearly visible in a normal COSY spectrum. These cross peaks allowed the location of all chemical-shift values. Unfortunately, the cross

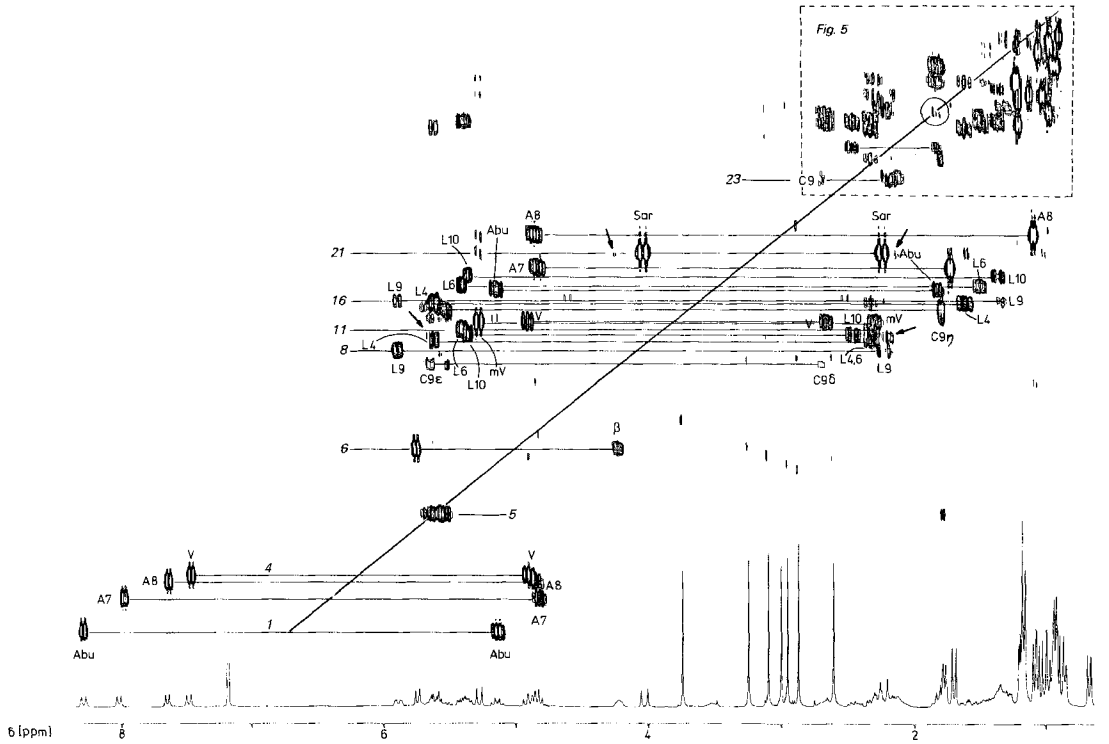


Fig. 4. *Double-Quantum-^{1}H-NMR spectrum (300 MHz) of* 1 *in* C_6D_6[2]). Coupled pairs are connected by horizontal lines and numbered with increasing DQ frequency (vertical axis).

Table 1. *Assignment of the Double-Quantum-^{1}H-NMR Spectrum (300 MHz)*[2])

Line No.	coupling	*Line* No.	coupling	*Line* No.	coupling
1	Abu-2 (NH,α)	*16*	MeLeu-9 (α,β_2)	*31*	MeLeu-6 (γ,β_2)
2	Ala-7 (NH,α)	*17*	Abu-2 ($\alpha,\beta_{1,2}$)	*32*	Val-5 (β,γ_2)
3	D-Ala-8 (NH,α)	*18*	MeLeu-6 (α,β_2)	*33*	MeLeu-9 (β_1,β_2)
4	Val-5 (NH,α)	*19*	MeLeu-10 (α,β_2)	*34*	MeVal-11 (β,γ_1)
5	MeBmt-1 (ε,ζ)	*20*	Ala-7 (α,β)	*35*	MeLeu-10 (γ,β_2)
6	MeBmt-1 ($\alpha\beta$)	*21*	Sar-3 ($\alpha_1\alpha_2$)	*36*	MeLeu-4 (β_2,γ)
7	MeBmt-1 (ε,δ)	*22*	D-Ala-8 (α,β)	*37*	MeLeu-10 ($\gamma,\delta_1/\delta_2$)
8	MeLeu-9 (α,β_1)	*23*	MeBmt-1 (α_1,α_2)	*38*	MeVal-11 (β,γ_2)
9	MeLeu-4 (α,β_1)	*24*	MeLeu-6 (β_1,γ)	*39*	Abu-2 (β,γ)
10	MeLeu-10 (α,β_1)	*25*	MeLeu-10 (β_1,γ)	*40*	MeLeu-9 (γ,β_2)
11	MeLeu-6 (α,β_1)	*26*	MeLeu-4 (β_1,β_2)	*41*	MeLeu-4 (γ,δ_1)
12	Val-5 (α,β)	*27*	Val-5 (β,γ_1)	*42*	MeLeu-4 (γ,δ_2)
13	MeVal-11 (γ,β)	*28*	MeLeu-10 (β_1,β_2)	*43*	MeLeu-9 (γ,δ_1)
14	MeBmt-1 (ζ,η)	*29*	MeLeu-6 (β_1,β_2)	*44*	MeLeu-9 (γ,δ_2)
15	MeLeu-4 (α,β_2)	*30*	MeLeu-4 (β_1,γ)		

peaks for the β,γ coupling appear near the diagonal or are covered by other cross peaks in the range of 1.1–2.4 ppm. Furthermore their intensities are very low. Attempts to solve these problems by a 90-45-COSY spectrum [16] [19] did not yield a satisfactory result. Hence we performed a two-quantum-^{1}H-NMR spectrum (= DQ spectrum = double-quantum spectrum [21–30]). We used the usual multi-quantum pulse sequence [22] with an observing pulse β longer than 90° [24].

$$90_\phi\tau - 180_\phi - \tau - 90_\phi - t_1 - \beta - t_2$$

$$\phi = x, y, -x, -y; \beta = x; \text{receiver} = +, -$$

This enhances the most interesting double-quantum peaks of coupled proton pairs in the echo signal (signals of type I [26]). The resulting DQ spectrum looks similar to the well known 2D-INADEQUATE [31–34] spectrum for C-atoms except that the signals expose a more complicated fine structure in f_2. Single- and multiple-quantum excitation other

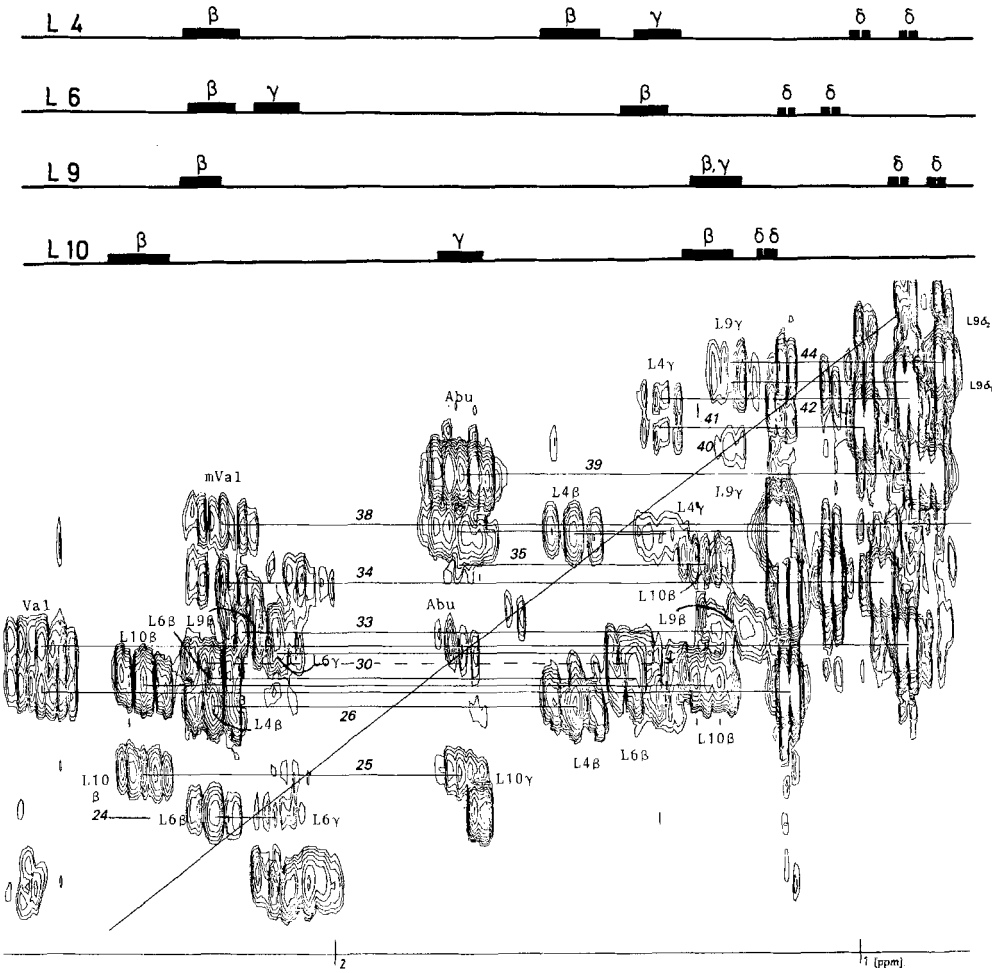


Fig. 5. *High-field part of the DQ-^{1}H-NMR spectrum of* Fig. 4 *and assignment of the 4 MeLeu residues of* **1**[2])

than DQ's are suppressed by the usual phase-cycling procedure [21] [35]. The 2D-DQ spectrum of **1** in C_6D_6, obtained with a β-pulse of 135°, is shown in *Fig. 4*. To emphasize large coupling constants, a τ-value of 10 ms was applied. This corresponds to an optimal DQ excitation for coupling constants of 25 Hz. The coupled pairs are connected by horizontal lines and numbered (in italics) with increasing DQ frequency (vertical axis). They are symmetric with respect to the line $\omega_1 = 2\omega_2$, which is also shown in *Fig. 4*. The analysis of the DQ spectrum is listed in *Table 1*.

Several advantages of such a DQ-^{1}H-NMR spectrum are obvious: (*i*) Strongly coupled systems, such as the two olefinic protons at 5.6 ppm (*Line 5*) and the signals, of the diastereotopic β-protons of Abu (between *Lines 31 and 32* in *Fig. 5*) are clearly indicated. In a COSY spectrum the cross peaks for this system appear close to the diagonal and are not easily observed. (*ii*) Singlets which are not coupled (*e.g.* the 7 *N*-methyl groups), are completely filtered out and do not cover small signals [36]. (*iii*) Coupling between protons is easily identified by symmetry to the $\omega_1 = 2\omega_2$ line even when one partner is rather broad because of coupling to many next neighbors.

These advantages were used to assign the β,γ connectivities of the 4 MeLeu residues. Signals for these couplings are clearly visible in the expanded region of the DQ spectrum (*Fig. 5*; L4: *Lines 30,36*; L6: *Lines 24,31*; L9: *Lines 33,40*; L10: *Lines 25,35*). The similar chemical shifts of L9(β) and L9(γ)[2]) are indicated in *Fig. 5* by the *Number 40*.

This example shows that DQ-^{1}H-NMR spectroscopy is an interesting complement to other homonuclear techniques. In addition, accidental overlap is reduced. It is the combination of several 2D techniques which improves the reliability of assignments in complex spectra dramatically.

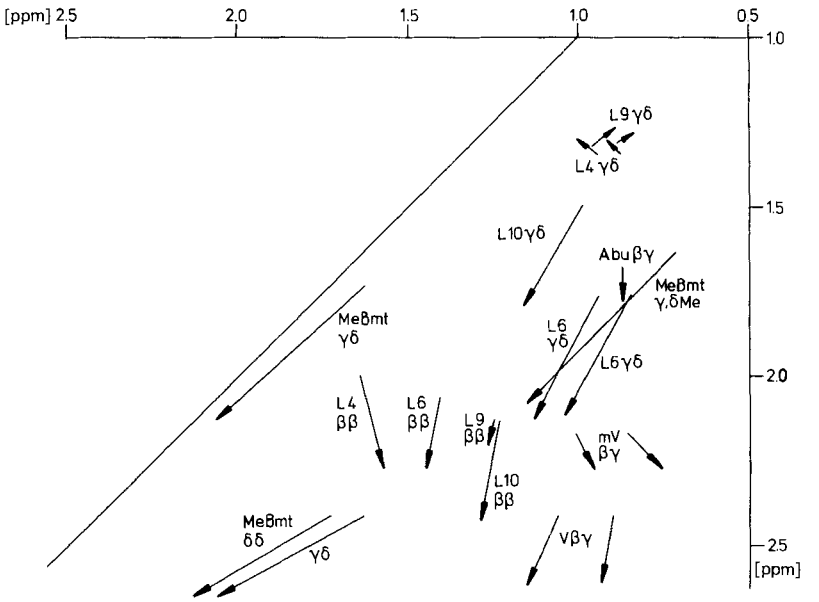


Fig. 6. *Aromatic-solvent-induced shift of* **1** *presented* via *arrows, which indicate the moving of the cross peaks from* $CDCl_3$ *to* C_6D_6[2]). Only the high-field region is shown.

Having analyzed the COSY spectrum in C_6D_6 and $CDCl_3$ (*Fig. 2* and *3*), the COSY spectra of $CDCl_3/C_6D_6$ mixtures allow the correlation of signals in both pure solvents. For this purpose we copied the spectra on transparencies which were arranged according to increasing C_6D_6 content between glas plates. Then it was easy to follow the movement of the cross peaks in the solvent mixtures. The result of this titration is shown in *Fig. 1* and *6*. Interestingly most ASIS effects are positive (low-field shifts with increasing amount of C_6D_6). Only both α-protons of Sar and the methyl groups of MeVal are shifted upfield.

The assignment of the *N*-methyl groups was done in combination with the sequence assignments (see below).

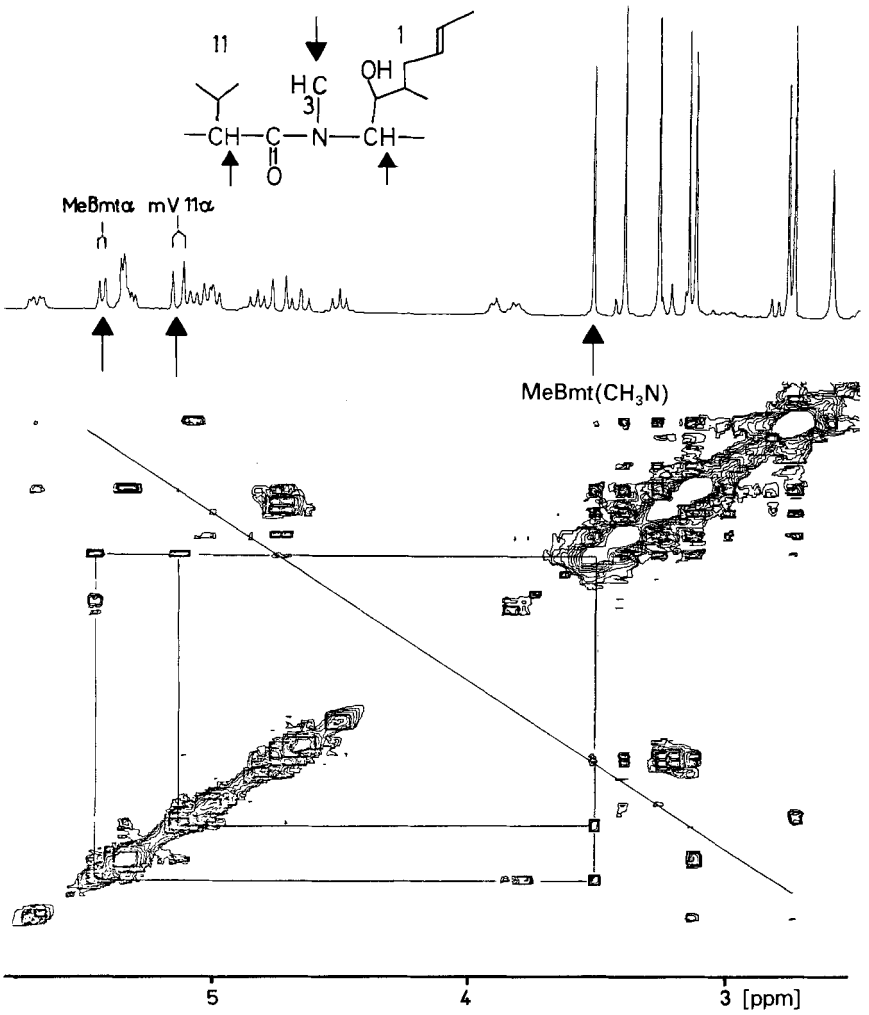


Fig. 7. *Part of the $^1H,^1H$-COSY-NMR spectrum (300 MHz) optimized for the detection of small couplings of* **1** *in $CDCl_3$*[2]). Two cross peaks to α-protons for each *N*-methyl group yield the assignment of the latter and connectivities in the peptide chain. Evaluation for the *N*-methyl group of MeBmt is demonstrated. The spectrum was symmetrized. Axial peaks at the carrier frequency at 4.2 ppm have been removed. Some additional artifacts lying at a line from the left upper corner to the right lower one are present.

2.3. *Sequence Assignments.* Scalar homonuclear couplings across amide bonds [38–43] are rarely used for peptide sequencing [40] [41] [43]. Two coupling pathways exist for *N*-methyl groups: the four-bond coupling (^4J) to the α-proton of the same amino acid and the five-bond coupling (^5J) to the α-proton of the sequentially preceding amino acid. Simultaneous appearence of both couplings enables an unequivocal proof for the connectivity of both amino acids as well as the assignment of the *N*-methyl group. A COSY spectrum with delays in evolution and detection period is especially well suited to follow such long-range coupling pathways. Such a spectrum in $CDCl_3$ is presented in *Fig. 7.*

As an example, the *N*-methyl group of MeBmt residue shows cross peaks to two α-protons, which are identified above *via* their affiliation to the spin systems of MeVal-11 and MeBmt. By the same arguments, the 5 low-field *N*-methyl signals in $CDCl_3$ are easy to assign. A similar spectrum in C_6D_6 enables a complete assignment of all *N*-methyl groups and of the peptide sequence *(Table 2)*.

Having assigned the *N*-methyl groups it is interesting to discuss their NOE effects. Only five NOE effects from *N*-methyl groups to α-protons across amide bonds are visible in a NOESY spectrum [44] [45]: mV(α)/MeBmt(CH_3N), Abu(α)/Sar(CH_3N), Val(α)/L6(CH_3N), A8(α)/L9(CH_3N), and low-field α of Sar/L4(CH_3N)[2]). Certainly these amide bonds are in a *trans*-conformation. On the other hand a strong NOE effect between the two α-protons of L9 and L10 indicates a *cis*-conformation about this amide bond. The high-field α-proton of Sar exhibits a small NOE effect to Sar(CH_3N). Only two amide protons show strong NOE effects to α-protons of the preceding amino acid (Abu(NH) to MeBmt(α); A7(NH) to L6(α)). Hence, NOE effects alone are not sufficient for a com-

Table 2. *Assignment of* N-*Methyl Groups* via *Cross Peaks to Protons in a* $^1H,^1H$-*COSY-NMR Spectrum Optimized for Small Coupling Constants*[a])

Residue No.	*N*-Methyl group δ [ppm]		Cross peak to α-proton δ [ppm]		Consequences	
	in $CDCl_3$	in C_6D_6	in $CDCl_3$	in C_6D_6	Assignment of the *N*-methyl groups	Assignments and connectivity[b])
1	3.51	3.73	5.45 (MeBmt); 5.15 (MeVal)	5.70 (MeBmt); 5.27 (MeVal)	MeBmt	
3	3.40	3.08	4.76 (Sar (α_1)); 5.03 (Abu)	5.12 (Abu)	Sar	
6	3.25	3.23	5.02 (MeLeu); 4.67 (Val)	5.39 (MeLeu); 4.89 (Val)	MeLeu-6	MeLeu-6
9	3.12	2.93	5.70 (MeLeu); 4.84 (Ala)	5.88 (MeLeu); 4.84 (Ala)	MeLeu-9	MeLeu-9 A8[c])
4	3.11	2.59	4.76 (Sar (α_1)); 5.34 (MeLeu)	4.01 (Sar (α_1)); 5.60 (MeLeu)		
11	2.71[e])	2.98	–	5.27 (MeVal)	MeVal-11	
10	2.70[e])	2.85	5.10 (MeLeu); 5.70 (MeLeu)	5.35 (MeLeu); 5.88 (MeLeu)	MeLeu-10	MeLeu-10[d])

[a]) See text.

[b]) The connectivity does not answer the sequence of the amino acids, but with knowledge of the sequence it allows the differentiation and assignment of the 4 MeLeu and the 2 A. The long-range H,C-coupling to the carbonyl C-atoms enable a full assignment without prior knowledge.

[c]) This enables the indirect identification of A 7.

[d]) This follows because MeLeu-9 is already assigned unequivocally *via* its coupling to A 8.

[e]) The assignment of the cross peaks is ambiguous because of strong signal overlap in $CDCl_3$.

Table 3. *^{1}H- and ^{13}C-NMR Chemical Shifts of Cyclosporin A* (**1**) *in $CDCl_3$ and C_6D_6*[a])

Residue No.	Amino-acid residue	Group	^{1}H-NMR				^{13}C-NMR	
			$CDCl_3$		C_6D_6		$CDCl_3$	C_6D_6
			2K	32K	2K	32K		
1	MeBmt	CH_3N	3.52	3.51		3.73	33.97 *q*	34.33
		CO	–	–	–	–	169.65 *s*	170.73
		H–C(α)	5.45	5.47	5.7	5.72	58.75 *d*	59.96
		H–C(β)	3.82		4.21	4.20	74.74 *d*	74.95
		OH	3.87			3.5	–	–
		H–C(γ)	1.63		2.07		35.99 *d*	36.30
		H^1–C(δ)	2.41		2.65		35.63 *t*	36.04
		H^2–C(δ)	1.73		2.13			
		CH_3(δ)	0.72	0.71	1.15		16.76 *q*	18.30
		H–C(ε)	5.36		5.64		129.68 *d*	131.38
		H–C(ζ)	5.35		5.52		126.32 *d*	126.76
		CH_3(η)	1.62		1.75		17.96 *q*	18.69
2	Abu	NH	7.93	7.96		8.26	–	–
		CO	–	–	–	–	173.04 *s*	174.29
		H–C(α)	5.03		5.12	5.12	48.86 *d*	49.54
		H–C(β)	1.6–1.74		1.79		25.06 *t*	26.11
		H–C(γ)	0.87		0.89		9.93 *q*	10.73
3	Sar	CH_3N		3.40		3.08	39.40 *q*	39.58
		CO	–	–	–	–	170.50 *s*	171.80
		H–C(α)	4.76	4.74		4.01	50.37 *t*	50.10
			3.23			2.21		
4	MeLeu	CH_3N		3.11		2.59	31.32 *q*	31.39
		CO					169.35 *s*	170.21
		H–C(α)	5.34		5.60		55.51 *d*	56.20
		H^1–C(β)	2.00		2.27		35.99 *t*	37.01
		H^2–C(β)	1.64		1.58			
		H–C(γ)	1.44		1.42		24.90 *d*	25.79
		$CH_3(\delta_1)$	0.95		0.98		23.49 *q*	24.28
		$CH_3(\delta_2)$	0.88		0.91		21.18 *q*	22.70[b])
5	Val	NH	7.47	7.48		7.46	–	–
		CO	–	–	–	–	173.07 *s*	174.76
		H–C(α)	4.67	4.66		4.89	55.39 *d*	56.05
		H–C(β)	2.41		2.61		31.17 *d*	32.19
		$CH_3(\gamma_1)$	1.06		1.15		19.81 *q*	20.59
		$CH_3(\gamma_2)$	0.90		0.93		18.48 *q*	19.06
6	MeLeu	CH_3N		3.25		3.23	31.53 *q*	32.13
		CO	–	–	–	–	170.87 *s*	172.28
		H–C(α)	5.02		5.39	5.39	55.31 *d*	56.05
		H^1–C(β)	2.06		2.27		37.41 *t*	38.33
		H^2–C(β)	1.41		1.45			
		H–C(γ)	1.76		2.12		25.40 *d*	26.11
		$CH_3(\delta_1)$	0.94		1.13		23.87 *q*	24.16[b])
		$CH_3(\delta_2)$	0.85		1.05		21.93 *q*	21.93
7	Ala	NH	7.75	7.68		8.00	–	–
		CO	–	–	–	–	170.44 *s*	171.84
		H–C(α)	4.52	4.52		4.81	48.69 *d*	49.54
		H–C(β)	1.36	1.36		1.68	16.07 *q*	16.61

Residue No.	Amino-acid residue	Group	^{1}H-NMR				^{13}C-NMR	
			$CDCl_3$		C_6D_6		$CDCl_3$	C_6D_6
			2K	32K	2K	32K		
8	D-Ala	NH	7.18	7.17		7.63	–	–
		CO	–	–	–	–	172.87 *s*	174.80
		H–C(α)	4.84	4.83		4.84	45.20 *d*	45.83
		H–C(β)	1.26	1.26	1.06		18.19 *q*	18.46
9	MeLeu	CH_3N		3.12		2.93	29.65 *q*	29.95
		CO	–	–	–	–	169.75	171.04
		H–C(α)	5.70	5.70	5.88	5.87	48.30 *q*	48.95
		H^1–C(β)	2.13		2.19		39.04 *t*	40.25
		H^2–C(β)	1.25		1.26			
		H–C(γ)	1.32		1.28		24.70 *d*	25.48
		$CH_3(\delta_1)$	0.97		0.91		23.74 *q*	25.07[b])
		$CH_3(\delta_2)$	0.89		0.84		21.86 *q*	24.81
10	MeLeu	CH_3N		2.70		2.85	29.83 *q*	30.47
		CO	–	–	–	–	169.41 *s*	170.98
		H–C(α)	5.10		5.35		57.54 *d*	58.36
		H^1–C(β)	2.13		2.42	2.42	40.73 *t*	42.06
		H^2–C(β)	1.24		1.29			
		H–C(γ)	1.49		1.79		24.55 *d*	25.62
		$CH_3(\delta_1)$	0.98		1.17		23.85 *q*	24.34
		$CH_3(\delta_2)$	0.98		1.16		23.38 *q*	22.64[b])
11	MeVal	CH_3N		2.71		2.98	29.81 *q*	30.96
		CO	–	–	–	–	172.85 *s*	174.61
		H–C(α)	5.15	5.14	5.27		57.93 *d*	58.84
		H–C(β)	2.17		2.27		29.05 *t*	29.99
		$CH_3(\gamma_1)$	1.01		0.96		18.75 *q*	19.38
		$CH_3(\gamma_2)$	0.86		0.66		20.26 *q*	20.59

[a]) At 296 K in ppm from internal TMS. The ^{1}H-NMR data were obtained from a conventional 32K spectrum or from the cross-sections of a ^{1}H,^{1}H-COSY with 2K data points in f_2 at 300 MHz.

[b]) The signals of MeLeu-4(δ_2) and MeLeu-9 (δ_1) as well as those of MeLeu-6 (δ_1) and MeLeu-10(δ_2) in C_6D_6 may be exchanged[2]).

plete and unequivocal sequencing but do provide much important conformational information. Because of the difficulties in quantification of NOE cross peaks, we used one-dimensional NOE-difference spectra for the conformational discussion [6].

Sequencing *via* C,H couplings between CO and α-protons is described below in connection to the assignment of the CO groups.

The chemical-shift values of all protons in $CDCl_3$ and C_6D_6 are collected in *Tab. 3*.

3. Assignment of ^{13}C-NMR Spectra. – 3.1. *Aliphatic C-Signals.* In the broadband-decoupled ^{13}C-NMR spectra in $CDCl_3$ or C_6D_6, almost all 62 C-signals are resolved. Only a few signals overlap, but these split when the solvent is changed. All carbonyl C-atoms resonate in the range between 169 and 175 ppm. The 2 olefinic C-atoms of MeBmt are also in a typical range at 126–132 ppm, whereas 49 C-signals have to be assigned in the aliphatic region. The number of attached protons are obtained *via* the usual DEPT technique [46] [47]. A heteronuclear 2D-J,δ spectrum [48–52] was also performed in $CDCl_3$. The resulting multiplicities are given in *Table 3*.

C-Resonances of peptides are often assigned by comparison of their chemical shifts with those of isolated amino acids or model peptides. This procedure is justified in many cases, but when interesting conformational aspects are involved, signals may be shifted into an unexpected spectral range. Such an empirical procedure would obviously fail in the case at hand due to the fact that all the C(β)'s of the four Leu residues appear in a range from 35 to 42 ppm. The C(α)'s show an even larger splitting (9 ppm). We therefore used modern two-dimensional methods to assign the spectrum.

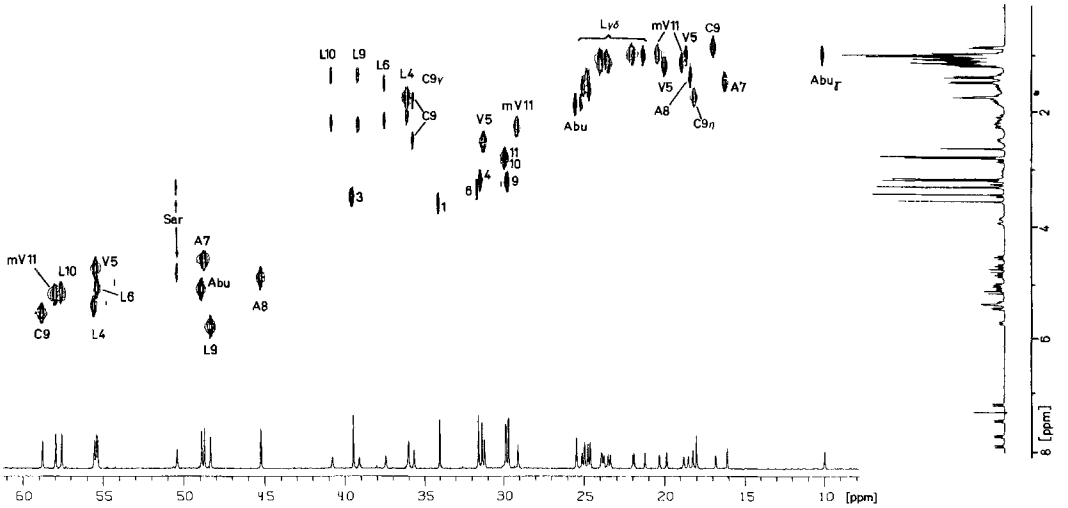


Fig. 8. *$^1H,^{13}C$-COSY-NMR spectrum (two-dimensional heteronuclear shift correlation) of* 1 *in* $CDCl_3$[2]). In part the amino-acid residues are abbreviated by their residue numbers.

With our knowledge of the proton chemical-shift values, a two-dimensional heteronuclear shift correlation *(Fig. 8)* enabled us to assign many of the C(α) and C(β) signals, but because of partial proton overlap again this experiment was performed in $CDCl_3$ *(Fig. 8)* and in C_6D_6. Aliphatic-C chemical shifts in these 2 solvents in general do not differ by more than 1 ppm (see *Table 3*, exception: MeBmt($CH_3(\delta)$ and $CH_3(\eta)$), MeLeu-10(β))[2]). The characteristic ASIS effects of H chemical shifts can now be used to discriminate some of the ambiguities. As an example, the two C(β)'s of MeLeu-9 (39.0 ppm) and MeLeu-10 (40.7 ppm) bear protons of similar chemical-shift values in $CDCl_3$ (H^1–C(β) at 2.13 and H^2–C(β) at 1.25 ppm, see *Table 3*), but in C_6D_6 the differing proton shifts (MeLeu-9: H^1–C(β) at 2.19, H^2–C(β) at 1.26; MeLeu-10: H^1–C(β) at 2.42, H^2–C(β) at 1.29 ppm) allow a clear distinction between the C(β)-signals (MeLeu-9 at 40.2 and MeLeu-10 at 42.1 ppm).

The shift correlations allowed the assignment of all C(α)'s, the olefinic C-atoms, all C(β)'s (except Ala und D-Ala), the *N*-methyl groups and $CH_3(\eta)$ of MeBmt. The remaining problem was the assignment of signals in the high-field spectral range (9–26 ppm) in which all C(γ)'s and all *C*-methyl groups resonate. Also the position of C(γ) of MeBmt (in $CDCl_3$ at 36.0 ppm) was not clear at this stage. An outstanding experiment for such assignment problems is provided by the relayed heteronuclear shift correlation [52–57]. Such a spectrum, obtained with our improved pulse sequence [56] [57], is shown for cyclosporin A in *Fig. 9*.

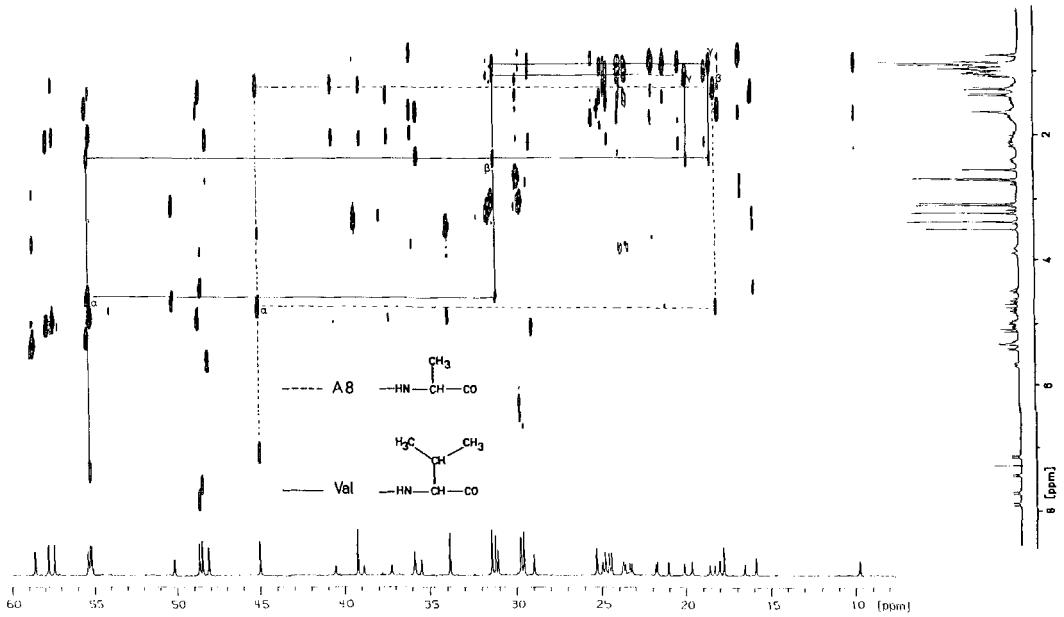


Fig. 9. *Relayed $^1H,^{13}C$-COSY-NMR spectrum of* **1** *in $CDCl_3$*). Assignment of A8 and Val is given as an example. Axial peaks at the proton carrier frequency 4.2 ppm and easy-to-assign mirror peaks of strong COSY peaks to this carrier frequency are removed.

The special feature of a relayed spectrum is the appearance of cross peaks for one C-atom not only at the chemical shift of the proton attached to that C-atom (C–H), but in addition cross peaks (so called relayed peaks) occur for protons which are homonuclearly coupled to that directly attached proton (C–H–H). This is demonstrated for Val in *Fig. 9*. In the H-chemical-shift range between 7 and 8 ppm, only the relayed peaks of the 4 NH protons to their C(α)'s are visible. Hence, the latter are directly identified. The middle one of the 3 C-signals around 55 to 56 ppm shows relayed peaks to one NH (7.47 ppm) and one β-proton (2.41 ppm). A horizontal line (at $\delta_H = 2.41$) connects this relayed peak and the shift correlation peak ($\delta(C(\beta))$ 32.2 ppm) as well as the two relayed peaks of the γ-methyl groups ($\delta(C(\gamma))$ 19.8, 18.5 ppm). The corresponding other relayed peaks are also observed. This spectrum yields the assignment of the kind of amino acid (Val) together with the identification of the C-signals. Of course, discrimination of A7 and A8 requires the knowledge of the sequence assignment in the ^{1}H-NMR spectrum, but this has already been done (see above). The C(β) of Abu was not directly evident from the relayed spectrum because one of the relayed signals ($\delta(C(\beta))$ 25.1; $\delta(H–C(\alpha))$ 5.03 ppm) was missing. Fortunately the characteristic multiplicity (t) of the C-resonance at 25.1 ppm in the heteronuclear 2D-J,δ spectrum allowed a clear differentiation from other signals with similar proton chemical shifts. The Abu γ-methyl group is then easily identified.

Signal assignments of the residual C-atoms C(γ) and $CH_3(\delta)$ of MeBmt are possible *via* relayed peaks between β and γ ($\delta(C(\beta))$ 74.7, $\delta(H–C(\gamma))$ 1.63 and $\delta(C(\gamma))$ 36.0; $\delta(H–C(\beta))$ 3.82 ppm) and γ and δ ($\delta(C(\gamma))$ 36.0; $\delta(CH_3(\delta))$ 0.72 and $\delta(CH_3(\delta))$ 16.8; $(H–C(\gamma))$ 1.63 ppm). The $CH_2(\delta)$ is characterized from the shift correlation and a weak relayed peak between γ and δ ($\delta(C(\gamma))$ 36.0; $\delta(H^1–C(\delta))$ 2.41 ppm).

The remaining problem is the complete assignment of the C(γ)'s and C(δ)'s of the 4 MeLeu's. The 4 C(γ)'s resonate in the range of 24 to 26 ppm. Their identification is facilitated by the multiplicity obtained by a DEPT spectrum and a heteronuclear J,δ spectrum. The assignment to the 4 different MeLeu residues follows from the shift correlation and weak relayed peaks to the C(β)'s *(Fig. 10)*. The relayed peaks at the γ-proton chemical shift on the position of the C(δ) signal enable us to find the connectivities between the C(γ)'s and the methyl groups attached to them. These peaks are indicated in *Fig. 10* by horizontal arrows at the resonances of their γ-protons. The assignment of the low field $CH_3(\delta)$ signals of MeLeu-6, -9, and -10 at *ca.* 24 ppm still bears ambiguities because of limited resolution at 300 MHz.

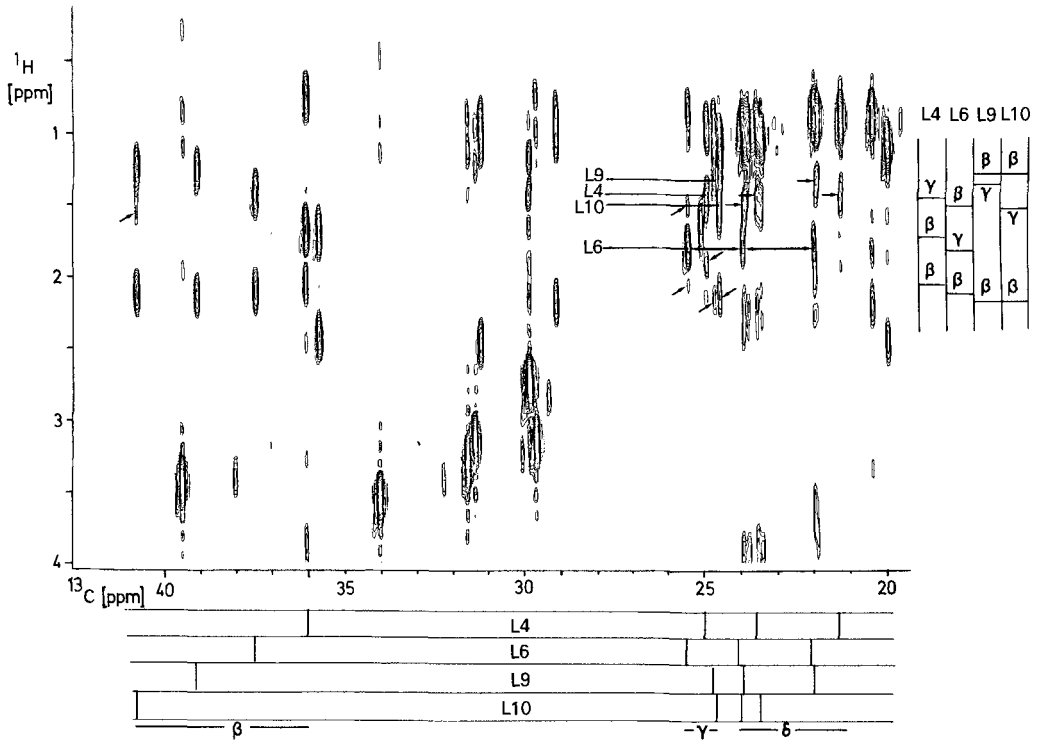


Fig. 10. *High-field part of the relayed $^1H,^{13}C$-COSY-NMR spectrum of* Fig. 2[2]). The assignments of the MeLeu signals are given. C,H-shift correlation peaks of CH(γ) of all MeLeu residues are indicated in the spectrum. Relayed peaks from the γ-proton to both C(δ)'s are indicated by horizontal arrows. Inclined arrows assign relayed peaks from β-protons to the C(γ)'s.

A recently recorded $^1H,^{13}C$-COLOC (heteronuclear correlation *via* long-range coupling) spectrum [58–60] at 500 MHz yielded the desired information. The $^1H,^{13}C$-COLOC exposes H,C-long-range couplings ($^2J_{C,H}$, $^3J_{C,H}$) which can be helpful to detect H-resonances at the position of the not directly bound C-signals. A sequence

$$90°(^1H),t_1/2,180°(^1H,^{13}C),\Delta_1\text{-}t_1/2,90°(^1H,^{13}C),\Delta_2,\text{acq.}(^{13}C,BB^1H)$$

with delays optimized by INEPT to $\Delta_1 = 27$ ms and $\Delta_2 = 37$ ms was applied for this purpose. The most interesting information results from the resonances at the δ-methyl

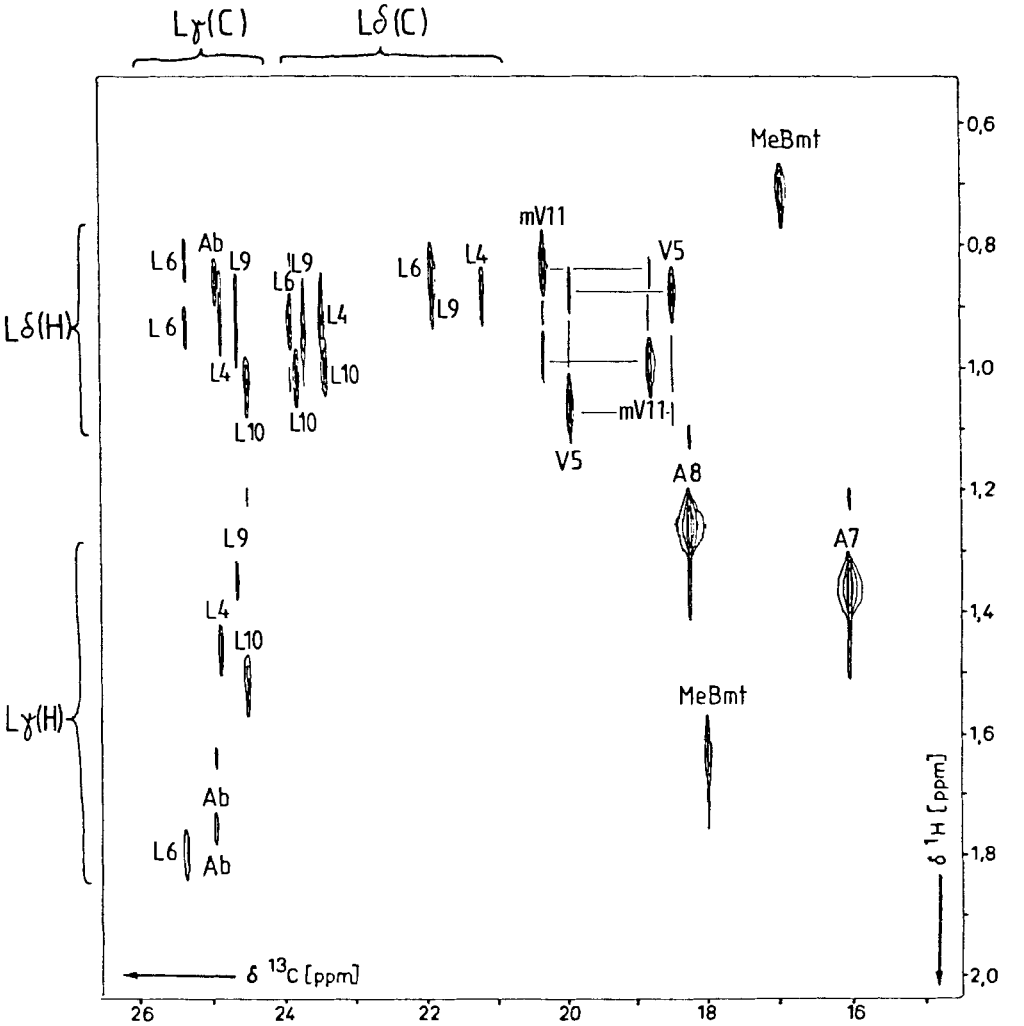


Fig. 11. *High-field part of the contour plot of a* $^{1}H,^{13}C$*-COLOC-NMR spectrum of* **1** *(500 mg) in* $CDCl_3$ *(2.5 ml)*[2]. ^{1}H-frequency 500 MHz. The cross peaks of MeVal-11 and Val are connected by lines.

proton ($CH_3(\delta)$) chemical shifts detected at the f_2-chemical shifts of the C(β) and C(γ) *carbon* signals (*Fig. 1* and *3* in [60]). *Fig. 11* exhibits the high-field part of the COLOC spectrum (^{13}C: 16–26 ppm) containing all MeLeu(γ) and MeLeu(δ) assignments. For example, the C(γ) signal of MeLeu-6 at 25.40 ppm exhibits cross peaks at the H-chemical shifts of the γ-proton (1.76 ppm) and the two δ-protons (0.94 and 0.85 ppm). Unfortunately methyl C-signals normally do not show cross peaks due to $^{2}J_{C,H}$ or $^{3}J_{C,H}$ couplings. An exception to this is shown for the coupling between two geminal methyl groups in MeVal-11 and Val-5.

3.2. *Carbonyl Resonances.* Carbonyl chemical shifts of peptides have so far attracted little attention [48]. This results partly from the difficulty of correlating molecular structure to chemical-shift data. Furthermore, carbonyl resonances appear in a rather narrow

chemical-shift range and are often not completely resolved. We present here the application of 2D-shift correlation for complete assignment of the 11 CO signals. Additionally this technique yields a sequence analysis and can be used for peptide sequencing in favorable cases. After completion of this work, the same technique was also reported elsewhere [62]. More recently we developed the $^{1}H,^{13}C$-COLOC experiment as a more convenient way to assign CO signals [58].

Carbonyl resonances in peptides of a fully coupled ^{13}C-NMR spectrum exhibit relatively broad signals due to couplings to N*H*, α- and β-protons in the range up to 9 Hz [63] [64]. Hence, we used a 2D-heteronuclear shift correlation optimized for 5 Hz *(Fig. 12)*. Cross peaks at each *C*O for up to 4 kinds of protons are seen: N*H* or C*H*$_3$N, 2 α-protons, and 1 β-proton. These 2 α-protons obviously must belong to attached amino acids connected by the CO-containing amide bond. In all but one case (A8(*C*O), Me-Leu(*H*–C(8α))[2]) both α-cross peaks appear, but that one missing cross peak is found at lower plot levels as well as in the cross section. Consequently, at the chemical shift of each

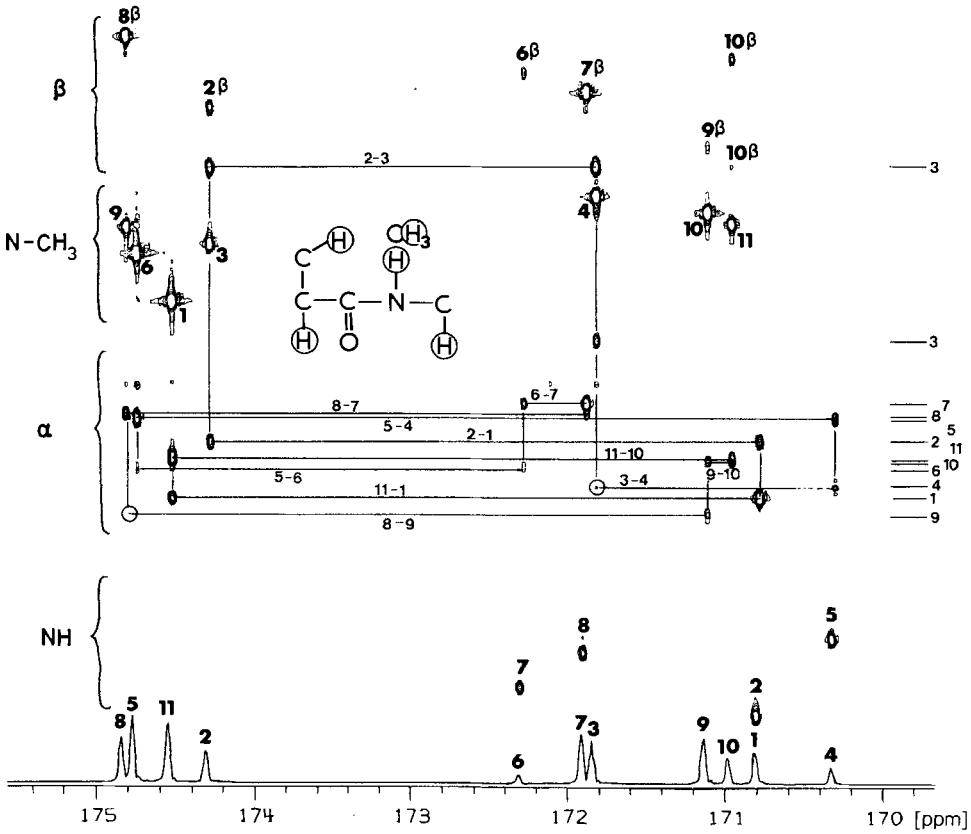


Fig. 12. *$^{1}H,^{13}C$-COSY-NMR spectrum of* **1** *optimized for 5-Hz proton coupling constants of the carbonyl C-signals in* C_6D_6. Shift-correlation peaks from both α-protons to the CO's enable the CO and the sequence assignments. Cross peaks yield also the proton assignment of the *N*-methyl groups. Connecting lines are drawn to indicate the way of sequence analysis of a peptide *via* such a spectrum. The amino-acid residues are abbreviated by their residue numbers.

α-proton resonance two cross peaks appear which give the connectivity to two neighboring CO groups. Thus one is able to follow the peptide sequence as indicated in *Fig. 12*.

Four strong cross peaks for the geminal arrangement *C*O-N*H* appear whereas no signals are observed for N*H* of the same amino acid. Obviously, the coupling constant of this three-bond coupling *H*NC(α)*C*O is too small [63] for satisfactory magnetization transfer.

The cross peaks of all 7 *N*-methyl groups are the strongest signals in the spectrum *(Fig. 12)*. Their proton chemical shift (vertical axis) can be used as a cross check of their assignment by small H,H couplings (see above). Some cross peaks caused by β-protons at the chemical shift of the CO signals of the same amino acid are indicated in *Fig. 12*. Their observation is sufficient to assign directly the CO resonances. Those CO's then might be used as a starting point in the sequential analysis.

The technique proposed here yields not only the connectivity between all amino acids but also the direction of the connectivities *i.e.* the sequence, because the coupling to N*H* or N-C*H*$_3$ occurs only to the amino acid following in the sequence. The application of long-range H,H couplings, on the other hand, yields only information about pairs of amino acids but not about the sequence.

4. Assignments of ^{15}N-NMR Spectra. – A H-decoupled ^{15}N-NMR spectrum shows 11 N-signals *(Table 4)*. The four *N*H groups are easily identified *via* an INEPT spectrum. The assignment of these 4 N-signals was performed *via* a ^{1}H,^{15}N-COSY spectrum

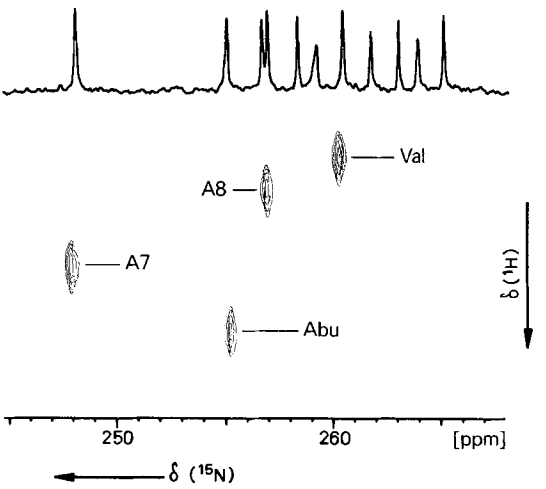


Fig. 13. *^{1}H,^{15}N-COSY-NMR spectrum of* **1** *in C_6D_6*

Table 4. *^{15}N-NMR Chemical Shifts of* **1**. δ in ppm from $NH_4{}^{15}NO_3$ in an external capillary.

in $CDCl_3$	in C_6D_6	in $CDCl_3$	in C_6D_6
−248.8 A7	−248.1 A7	−259.2	−260.4 Val
−255.5 Abu	−255.1 Abu	−260.1	−261.8
−256.9	−256.7	−261.8	−263.1
−257.3 A8	−256.9 A8	−264.2	−263.9
−258.0	−258.3	−265.2	−265.1
−258.6 Val	−259.2		

(Fig. 13) [52] [53]. Unfortunately proton relaxations were too short to allow an assignment of the residual N-signals *via* $^1H,^{15}N$-COSY optimized for H,N-long-range coupling constants.

5. Conclusion. – The combination of different homo- and heteronuclear two-dimensional NMR experiments enabled us to assign all H- and C-resonances of cyclosporin (**1**). This assignment is based solely on scalar couplings by a minimum of empirical arguments. The remaining problem is the discrimination of *pro-R* and *pro-S* positions in geminal methyl groups and geminal protons of methylene groups. This has been accomplished by the discussion of chemical-shift considerations as well as by NOE effects. Because these parameters can only be understood in connection with the conformation of the molecule, we defer discussion of this problem to the subsequent paper.

We gratefully acknowledge financial support from the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie*.

6. Measurement Conditions. – 6.1. *General.* The one-dimensional ^1H-NMR spectra of *Fig. 1* and the NOESY spectra were recorded on a *WH 270* spectrometer (ν_o (^1H) 270 MHz) with an *Aspect 2000* computer *(Bruker Analytik)*, the $^1H,^{13}C$-COLOC spectrum was measured on an *AM 500* ($\nu_o(^{13}C)$ 125.7 MHz) spectrometer with an *Aspect 3000* computer *(Bruker)*; all other spectra were measured on a *Nicolet NT 300 WB* spectrometer ($\nu_o(^1H)$ 300 MHz; $\nu_o(^{13}C)$ 75.4 MHz; $\nu_o(^{15}N)$ 30.4 MHz) with a *NMC 1280* computer *(Nicolet Instruments)*. All measurements were performed at r.t., if not otherwise stated. Concentrations: ^1H-NMR spectra: 0.104M in $CDCl_3$, 0.045M in C_6D_6. ^{13}C-NMR spectra: 0.13M in $CDCl_3$, 0.066M in C_6D_6.

6.2. *One-Dimensional ^1H-NMR Spectra.* a) In $CDCl_3$: size 32K, sweep width 11904.76 Hz, pulse length 1.4 μs, 328 acquisitions. b) In C_6D_6: size 32K, sweep width 2994.012 Hz, pulse length 4.5 μs, 224 acquisitions.

6.3. *$^1H,^1H$-COSY Spectrum in $CDCl_3$.* Sequence: delay-90°-t_1-90°-t_2. Delay: 1.5 s, 90° pulse 6.9 μs, acquisition time 212.93 ms, spectral width in f_1 and f_2 2403.8 Hz, size 1K, 48 acquisitions, 512 increments, quad detection in both dimensions, sine-bell multiplications in both dimensions, zero filling in t_1.

6.4. *$^1H,^1H$-COSY Spectrum in C_6D_6.* Delay: 2.5 s, 90° pulse 12.0 μs, acquisition time 177.15 ms, spectral width in f_1 and f_2 2890.16 Hz, size 1K, 64 acquisitions, 256 increments, quadrature detection in both dimensions, sine-bell multiplications in both dimensions, zero filling in t_1.

6.5. *$^1H,^1H$-Double-Quantum Spectrum.* Sequence: delay 1–90°-delay 2–180°-delay 2–90°-t_1–135°-t_2. Phase cycling according to [13] was used. 90° pulse 10.8 μs, delay 1 3.5 s, delay 2 10 ms, acquisition time 454.66 ms, size 4K, spectral width in f_1 and f_2 4504.5 Hz, 40 acquisitions, 256 increments, sine-bell multiplications in both dimensions, double-zero filling in t_1. The spectrum was recorded in single-phase detection in both dimensions, only 1 of the 4 spectra is shown.

6.6. *$^1H,^1H$-COSY Spectra of the Solvent Titration.* 90° pulse length 6.65 μs, relaxation delay 1.4 s, size 2K, sweep width in both dimensions 2604.16 Hz, acquisition time 393.22 ms, quad detection in both dimensions, sine-bell multiplications in both dimensions. Two spectra in pure solvents and 5 spectra in solvent mixtures containing 6, 17, 28, 38, and 50 vol-% of C_6D_6 were recorded. Internal standard: TMS.

6.7. *$^1H,^1H$-COSY Spectrum with Delay in $CDCl_3$.* Sequence: delay 1–90°-delay 2-t_1–60°-delay 2–t_2. Relaxation delay 1.1 s, delay 2 200 ms, 90° pulse length 12.1 μs, spectral range in both dimensions 2600.16 Hz, acquisition time 196.61 ms, size 1K, 160 acquisitions, 128 increments, quad detection and sine-bell multiplications in both dimensions, two times zero filling in t_1, symmetrization of the data matrix.

6.8. *$^1H,^1H$-COSY Spectrum with Delay in C_6D_6.* 90° pulse length 11.1 μs, sweep width in both dimensions 2890.16 Hz, acquisition time 177.15 ms, delay 1 1.8 s. All other parameters as in the $^1H,^1H$-COSY spectrum in $CDCl_3$.

6.9. *One-Dimensional ^{13}C-NMR Spectra.* Relaxation delay 0.5 s, sweep width 15151.5 Hz, size 16K, zero filling to 32K was applied, pulse length 6 μs (22°).

6.10. *$^1H,^{13}C$-COSY Spectrum in $CDCl_3$.* Sequence: 90° (^1H)-$t_1/2$–180°(^{13}C)-$t_1/2$–Δ_1–90°($^1H,^{13}C$)-Δ_2-t_2. Relaxation delay 1.2 s, 90° pulse length ^1H: 28 μs, ^{13}C: 25 μs, Δ_1 4 ms, Δ_1 4 ms, Δ_2 2.7 ms, spectral range in f_2 10204.15 Hz (0–136 ppm), in f_1 2475.24 Hz (0–0.25 ppm), quad detection in both dimensions, 4 dummy scans, 192 acquisitions, 128 increments. Acquisition time 200.7 ms, size 4 K. Sine-bell multiplication and zero filling in t_1.

6.11. *Relayed $^1H,^{13}C$-COSY Spectrum in $CDCl_3$.* Sequence: 90°(^{1}H)-t_1/2–180°(^{13}C)-t_1/2–90°(^{1}H)-D1,D2–180°(^{1}H)-D1–180°(^{13}C)-D2–90°(^{1}H,^{13}C)-D3-t_2(BB^{1}H). Relaxation delay 1 s, D1 13.84 ms, D2 1.76 ms, D3 2.6 ms. 688 scans, 64 acquisitions, sine-bell multiplication in both dimensions, zero filling in t_1. All other parameters as described in the ^{1}H,^{13}C-COSY experiment.

6.12. *$^1H,^{13}C$-COSY Spectrum in C_6D_6.* Relaxation delay 1.4 s, 90° pulse length ^{1}H: 49 µs, ^{13}C: 23 µs, spectral range in f_2 10101 Hz (0–134 ppm), in f_1 2000 Hz (0–6.7 ppm), 192 acquisitions. All other parameters as described in the ^{1}H,^{13}C-COSY in $CDCl_3$.

6.13. *$^1H,^{13}C$-COSY Spectrum Optimized for 5-Hz Coupling Constants of the CO Signals.* Relaxation delay 1.5 s, 90° pulses ^{1}H 30 µs, ^{13}C 25 µs, Δ_1 100 ms, Δ_2 50 ms, spectral range in f_2 1000 Hz (only for the detection of the CO resonances between 169 and 173 ppm), in f_1 2645.5 Hz (whole proton spectral range), size 2K, acquisition time 1.02 s, 160 acquisitions, 8 dummy scans, 256 increments, quad detection in both dimensions, zero filling in t_1, sine-bell multiplication in t_1, exponential multiplication with LB = 1 in t_2.

6.14. *$^1H,^{15}N$-COSY Spectrum in C_6D_6.* The *Nicolet* horizontal probe (5 ml) was used. The pulse sequence as in the ^{1}H,^{13}C-COSY was used. Relaxation delay 1.0 s, 90° pulse length ^{1}H 34.6 µs, ^{15}N 22 µs, $\Delta_1 = \Delta_2 = 5.4$ ms, spectral range in f_2 2000 Hz, in f_1 1000 Hz (5.5–8.8 ppm), size 2K, acquisition time 512 ms, 512 acquisitions, 64 increments, quad detection in both dimensions, exponential multiplication LB = 2 in t_2, double exponential multiplication DM = 6 in t_1, zero filling in t_1.

6.15. *$^1H,^{13}C$-COLOC Spectrum in $CDCl_3$.* Applied to aliphatic C-atoms. Sequence: 90° (^{1}H), t_1/2,180°(^{1}H,^{13}C), (Δ_1-t_1/2),90°(^{1}H,^{13}C),Δ_2,acq. (^{13}C,BB^{1}H). Relaxation delay 1s, 90° pulse length ^{1}H: 50 µs, ^{13}C: 22.5 µs, Δ_1 27 ms, Δ_2 37 ms optimized to suppress direct $^1J_{C,H}$ of about 135 Hz, spectral range in f_2 7692 Hz (only for the detection of resonances between 10 and 60 ppm), in f_1 4545 Hz (full range), size 8 K, acquisition time 0.266 s, 192 acquisitions, 240 increments, quad detection in both dimensions, zero fillling in t_1 to 512 w, in t_2 to 8 K. Sine-bell multiplication in t_1, exponential multiplication (LB = 2) in t_2.

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