Peptide Conformations. 28.[1] Relayed Heteronuclear Correlation Spectroscopy and Conformational Analysis of Cyclic Hexapeptides Containing the Active Sequence of Somatostatin

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Abstract: Homo- and heteronuclear two-dimensional NMR techniques have been used to assign the ^1H and ^{13}C NMR spectra of the cyclic hexapeptides *cyclo*[Phe-D-Trp-Lys(Z)-Thr-Xxx-Pro] (**1**, Xxx = Gly; **2**, Xxx = Phe) in Me_2SO. Unequivocal assignments of Trp and Phe carbon resonances in **2** could only be obtained by heteronuclear relayed spectroscopy. Conformational analysis of **2** proves a βII' turn of the Phe[7]-CO←HN-Thr and a βVI turn for Thr-CO←HN-Phe[7] including a cis peptide bond between Phe[11] and Pro. Substitution of Phe[11] by Gly results in a dramatic change in the conformation: two species are observed in slow equilibrium (70:30). The major isomer involves a trans Gly-Pro bond. The technique of relayed heteronuclear correlation spectroscopy and a new, improved pulse sequence are described, which yield an increase in sensitivity of $2^{1/2}$ to 2 depending on the chemical shift of the protons. This gain is also essential in many nonpeptide applications, e.g., to natural products, to rare materials, or in case of low solubility. The relative merits of various techniques to establish the sequences of carbon nuclei in the molecular backbone are discussed.

Conformational analysis of peptides by NMR spectroscopy requires a careful and reliable assignment of carbon, proton, and, if possible, nitrogen signals.[2] In this respect, two-dimensional NMR spectroscopy (2D NMR)[3] provides great advantage over conventional NMR methods. We demonstrate in this communication that the "relayed spectroscopy"[4-6] can provide unique information leading to a clear-cut picture of proton and carbon connectivities. Furthermore an improved new pulse sequence of "relayed spectroscopy" is presented, which results in a significant sensitivity gain. The technique is demonstrated on two cyclic hexapeptides, *cyclo*[Phe-D-Trp-Lys(Z)-Thr-Xxx-Pro] (**1**; Xxx = Gly; **2**, Xxx = Phe). The peptide **2** is the protected derivative of the cyclohexapeptide that has found considerable scientific and pharmaceutical interest due to its high somatostatin activity.[7] We have assigned for **1** and **2** all proton and carbon signals (with the exception of some aromatic resonances) by the combination of modern NMR techniques. This provides a reliable basis for the interpretation of spectral parameters to study the conformation of both peptides.

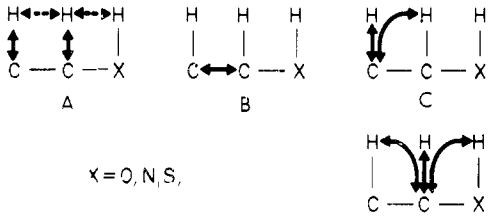
Determination of Carbon and Proton Connectivity by Advanced NMR Techniques

Proton and carbon coupling constants are probably the most informative sources to deduce connectivities in molecular frameworks. Organic chemists are especially interested in carbon connectivities to elucidate the molecular skeleton. In simple cases the analysis can be performed by inspection of conventional proton or carbon spectra. For unequivocal assignments in more complex situations, however, it is necessary to use advanced two-dimensional (2D) spectroscopy techniques.

Three different types of techniques have been proposed to elucidate carbon and proton connectivity: (A) heteronuclear 2D carbon-proton shift correlation combined with homonuclear proton 2D correlation spectroscopy, which provides an indirect way to determine carbon connectivity; (B) Direct detection of carbon-carbon couplings by the INADEQUATE technique; (C) detection of remote carbon-proton connectivity by relayed spectroscopy. We may represent the three types of connectivity detected by techniques A, B, and C by the diagrams shown in Chart I.

In recent years, heteronuclear 2D shift correlation spectroscopy[8-11] has become an accepted method to facilitate the assignment of both ^{13}C and ^1H NMR spectra of complex molecular systems.[12-18] In the most widely used form of the technique,[9,12] the information about chemical shifts is transmitted from ^1H to ^{13}C nuclei almost exclusively through one-bond $^1J_{CH}$ couplings. In the two-dimensional spectra, the frequency coordinates (ω_1, ω_2) correspond to the chemical shifts δ^H and δ^C of individual CH_n groups in the molecule. The usual heteronuclear 2D experiment does not provide information about the sequence of these CH_n groups in the molecular backbone. For an unequivocal deter-

Chart I



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mination of the connectivity of the CH_n groups it is necessary to identify J_{HH} or J_{CC} couplings or to utilize nuclear Overhauser effects.

Very recently, novel experiments have been proposed[4-6] that involve two consecutive magnetization-transfer steps to relay information between nuclei that are not coupled together but possess a common coupling partner. In heteronuclear systems, relayed magnetization-transfer experiments first bring about a transfer from one proton to another through a homonuclear coupling, and subsequently to ^{13}C through a one-bond heteronuclear coupling. Thus in a fragment of the type $C^AH_n^A-C^BH_m^B$ two distinct pathways are open to relayed magnetization transfer: $H^A \rightarrow H^B \rightarrow C^B$ and $H^B \rightarrow H^A \rightarrow C^A$, assuming a resolved vicinal $J_{H^AH^B}$ coupling (but without requiring long-range $^3J_{H^AC^B}$ and $^3J_{H^BC^A}$ couplings). In a relayed 2D spectrum, these two processes lead to peaks at ($\omega_1 = \delta_{H^A}$, $\omega_2 = \delta_{C^B}$) and ($\omega_1 = \delta_{H^B}$, $\omega_2 = \delta_{C^A}$), respectively, that appear as novel features *in addition* to the peaks observed in a conventional heteronuclear 2D spectrum, which stem from single-step magnetization transfer and appear at ($\omega_1 = \delta_{H^A}$, $\omega_2 = \delta_{C^A}$) and ($\omega_1 = \delta_{H^B}$, $\omega_2 = \delta_{C^B}$). These four signals appear at the corners of a rectangle in the 2D frequency domain.[6] The appearance of such a pattern of peaks, barring accidental overlaps, constitutes a proof that the two carbon signals stem from sites that must be in the immediate vicinity within the molecular framework.

Relayed spectroscopy provides similar (though not identical) information as double-quantum NMR of $^{13}C-^{13}C$ pairs in the so-called "INADEQUATE" method.[19-23] In the latter case, only molecules containing two ^{13}C isotopes contribute to the signal, whereas relayed spectroscopy makes use of all ^{13}C nuclei present at natural abundance and is therefore more sensitive.[24] On the other hand, the efficiency of relayed transfer decreases with increasing complexity of the proton coupling network, and the method does not reach the sensitivity of conventional 2D correlation spectroscopy.

Relayed 2D spectroscopy and the INADEQUATE method provide complementary information. INADEQUATE provides a direct mapping of the carbon–carbon connectivities. However the assignment of the connected CH_n fragments is based exclusively on carbon-13 chemical shifts. In relayed 2D spectroscopy, on the other hand, the information of the proton resonances and their assignment is efficiently utilized in assigning the carbon nuclei and in tracing their connectivity.

Relayed 2D spectroscopy is less susceptible to accidental overlap of proton or carbon-13 resonances than homonuclear proton correlation spectroscopy or than the INADEQUATE technique, because the two frequency axes refer to different spectra, thus minimizing the chance of overlap in both dimensions. Furthermore relayed spectroscopy can also be used to assign CH carbons attached to NH or OH (e.g., α-carbons of amino acids or the Thr β-carbon) even when the CH-proton shifts are not resolved.

An important aspect in comparing the three methods, as mentioned above, is their sensitivity, which decreases in the order A > C > B. Modern NMR spectrometers with a proton resonance frequency around 300 MHz require at least 0.02 M solution (0.05-mmol material) for a 2D $^1H-^{13}C$ shift correlation experiment (A) to be performed in 12 h. A relayed spectrum (C) requires a higher concentration for the same performance time, since the coherence transfer functions discussed below are smaller than unity. The sensitivity may be reduced further by relaxation during the longer pulse sequence, especially when small proton couplings are utilized for the transfer. Typically, about 4-times more substance is needed for a relayed spectrum than for a standard shift correlation spectrum. The INADEQUATE experiment requires a significantly larger amount of sample,[24] and often the solubility of the substance limits the application of this powerful method.

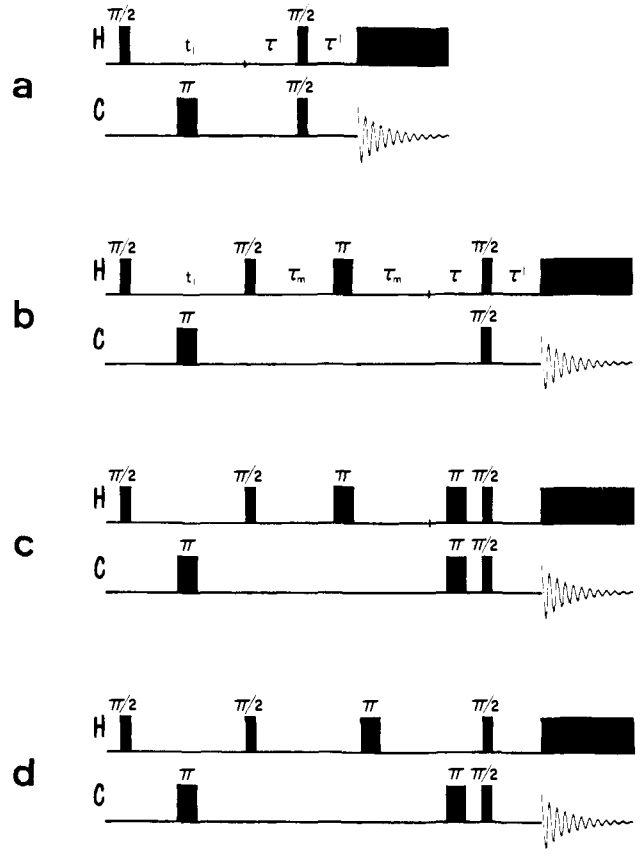

Figure 1. Pulse sequences for conventional and relayed two-dimensional heteronuclear shift correlation: (a) conventional heteronuclear shift correlation; (b) relayed heteronuclear shift correlation; (c) relayed technique with refocussing pulses; and (d) relayed technique with consolidated refocussing pulses.

Relayed Spectroscopy Technique

The pulse sequence for conventional heteronuclear correlation of the chemical shifts of protons and carbons is shown in Figure 1a. The mechanism of magnetization transfer has been discussed in detail elsewhere.[9,12] Suffice it to say that the delay $\tau \simeq (2J_{CH})^{-1}$ allows the ^{13}C satellites in the ^1H spectrum to acquire opposite phases in the rotating frame, which is a prerequisite for the transfer of magnetization by the $\pi/2$ pulses applied to both ^1H and ^{13}C nuclei. The subsequent τ' delay allows the antiphase ^{13}C magnetization to convert into in-phase magnetization before applying broad-band proton decoupling. The sequence in Figure 1a may be improved by inserting pairs of π pulses applied simultaneously to both ^1H and ^{13}C nuclei at the midpoints of both τ and τ' intervals: This is useful to remove frequency-dependent phase shifts when pure absorption mode spectra are desired.[25] However, it has no effect on spectra presented in the absolute-value mode, except for a slight gain in sensitivity due to refocusing of inhomogeneous decay.

To obtain relayed magnetization transfer, an additional $[\pi/2-\tau_m-\pi-\tau_m]$ sequence is inserted in the experiment, leading to the scheme shown in Figure 1b, recently described and successfully applied to aliphatic systems by Bolton.[6] In this scheme, the second $\pi/2$ proton pulse induces magnetization transfer between protons (for example $H^A \rightarrow H^B$), much as in homonuclear correlation spectroscopy.[3,26-28] The mixing interval $2\tau_m \leq (2J_{HH})^{-1}$

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allows the proton magnetization to evolve under the effect of homonuclear couplings alone, unaffected by shifts or heteronuclear couplings. In the case of a simple C^AH^A–C^BH^B fragment, the net magnetization transfer $H^A \rightarrow H^B \rightarrow C^B$ is made possible because the H^B magnetization, initially in antiphase with respect to J_{AB}, yields an in-phase component at the end of the $2\tau_m$ interval that is proportional to sin $(2\pi J_{AB}\tau_m)$. In a $C^AH_m{}^A$–$C^BH_n{}^B$–$C^CH_k{}^C$ fragment, the transfer $H_m{}^A \rightarrow H_n{}^B \rightarrow C^B$ is most efficient when the $2\tau_m$ interval is chosen such as to optimize the transfer function[29] $f^{relay} = \sin(2\pi J_{H^AH^B}\tau_m)\cos^{(m-1)}(2\pi J_{H^AH^B}\tau_m)\cos^k(2\pi J_{H^BH^C}\tau)\exp(-2\tau_m/T_2)$. Normal magnetization transfer through one-bond couplings also occurs in the relayed experiment. In the same molecular fragment, the transfer $H_m{}^B \rightarrow C^B$ is governed by the transfer function $f^{direct} = \cos^m(2\pi J_{H^AH^B}\tau_m)\cos^k(2\pi J_{H^BH^C})\exp(-2\tau_m/T_2)$. This function is equal to unity for $\tau_m = 0$, i.e., when the relayed experiment is reduced in effect to a normal heteronuclear correlation experiment. The relative sensitivities of the two experiments are governed by the transfer functions f. In extended coupling networks, both expressions for f^{relay} and f^{direct} must include additional cosine factors for couplings to further nonparticipating protons. Generally, a compromise setting at $2\tau_m = (5J_{HH}{}^{av})^{-1} \simeq 28$ ms is satisfactory for most coupling networks. For the transfer function f^{relay}, assuming $m = n = k = 2$, $J_{HH} = 7$ Hz, and a proton line width of 2 Hz ($T_2 = 0.16$ s) one obtains $f^{relay} = 0.26$. This implies that, in comparison with conventional heteronuclear 2D, a 4-fold concentration is required in this common situation.

The subsequent period $\tau \simeq (2J_{CH})^{-1}$ has the same function in Figure 1b as in Figure 1a. Note that the phase of the magnetization at the beginning of the τ interval in the relayed experiment is common to all protons, since it is tied to the rf phase of the second proton $\pi/2$ pulse in the sequence. For this reason, the proton magnetization should not be allowed to precess freely in the τ period, since shift-dependent precession affects the efficiency of the magnetization transfer from ^1H to ^{13}C nuclei.

This problem can be alleviated by cycling the phase of the last proton $\pi/2$ pulse.[5,6] A more satisfactory approach consists of removing the effects of chemical shifts in the τ interval. This can be achieved with the improved sequence in Figure 1c, where a pair of π pulses applied to ^1H and ^{13}C nuclei is inserted in the middle of the τ delay, in a manner that closely resembles the "INEPT" sequence.[30,31] The resulting advantage in sensitivity with respect to the phase cycling technique varies between 2 and $2^{1/2}$ depending on the chemical shifts of the protons. Close inspection of the sequence in Figure 1c shows that the two π pulses applied to the protons can be consolidated[22] into a single π pulse applied in the middle of the $(2\tau_m + \tau)$ interval. The resulting sequence, shown in Figure 1d, was actually used in the experiments described in this paper. To reduce the risk of artifacts, the π pulses applied to the ^{13}C nuclei were replaced by $(\pi/2)_x\pi_y(\pi/2)_x$ pulse sandwiches,[32] while errors due to the π pulse applied to the ^1H nuclei were cancelled by phase cycling.[33] To achieve quadrature detection in both frequency domains (i.e., to be able to position both ^1H and ^{13}C carrier frequencies in the middle of the corresponding spectral ranges), the phases of the *first* proton pulse and the *last* carbon pulse were cycled through x, y, $-x$, and $-y$ while the signals were alternatively added and subtracted.[34,35] In heteronuclear 2D NMR spectroscopy the problem of sensitivity is always significant. Hence, the variation of the relayed technique presented in this paper is important for its application to larger molecules, to biological relevant molecules, or to materials of low solubility.

Assignment of ^{13}C and ^1H Signals in Cyclic Hexapeptides

Recent interest in the biological activity of somatostatin and its derivatives[36] led to the synthesis and conformational studies of cyclic peptides containing the active sequence[37-39] of somatostatin (Phe^7-D-Trp^8-Lys^9-Thr^{10}). In natural somatostatin a L-Trp is found in position 8. The numbering of the amino acid sequence given in this paper follows those of the natural product. The work of Veber et al. has demonstrated that inclusion of this sequence in cyclic and bicyclic structures retains the somatostatin activity.[40-45] We have synthesized a series of cyclic penta-[16] and hexapeptides[46] to perform conformational analysis and activity studies. In this paper we wish to report the NMR results of two closely related peptides, in which the active sequence is bridged by Phe-Pro^7 and Gly-Pro. We choose the ϵ-Lys-Z-protected peptides in Me_2SO because we have shown in several other peptides that the conformation of the peptide backbone is similar with or without the Z group. In our opinion the conformation in Me_2SO is probably of higher relevance to the conformation at the receptor side than the conformation of the hormone model in H_2O because the environment in the organic solvent is more similar to the lipid phase. (One of the referees does not agree with this point.) The meeting of the hormone with its receptor seems to involve a two-dimensional lateral diffusion in the membrane rather than a direct approach from the surrounding solution.[47-49] Thus the conformation of hormones in lipophilic media is of great importance.

The 270-MHz ^1H NMR spectrum of the Gly-Pro derivative **1** shows the presence of two conformations (70% **1a**, 30% **1b**). The dynamic equilibrium between the two conformers was established by the reversible coalescence phenomenon at temperatures higher than about 100 °C[50-52] and the observation of saturation transfer at room temperature in the course of double resonance experiments.[53] The major isomer includes a trans Gly–Pro bond, as was shown by the chemical shift values of the Pro β and γ signals (β, 29.0; γ, 24.0).[2] To arrive at this conclusion, it is necessary to distinguish Lys and Pro signals in this region. Conventional 2D ^1H–^{13}C shift correlation (Figure 2) can solve

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Table I. ^{1}H NMR Data of the NH Protons of Cyclic Hexapeptides in Me_2SO

		D-Trp[8]	Lys[9]	Thr[10]	Xxx[11] [d]	Pro[6]	Phe[7]
δ (ppm)	1a	7.77	8.32	7.16[a]	7.43		7.48
	2	8.41	8.65	6.95[a]	8.30		7.21[a]
$10^3\Delta\delta/\Delta T$, ppm K^{-1}	1a	4.0	3.5	−0.2[a]	0.0		2.9
	2	4.8	5.0	0.2[a]	2.8		1.0[a]
	3	5.6	6.1	0.3	3.7		1.6
$^3J_{HNC_\alpha H}$, Hz	1a	6.2	7.7	8.8[a]	8.3[c]		8.5
	2	7.6	6.6	6.9[a]	5.5		5.8[a]
	3[b]	4.9	5.9	9.2	2.2		7.8

[a] NH signals covered by aromatic resonances were determined by difference spectroscopy. [b] In D_2O. [c] $^3J_{AX} + {}^3J_{BX}$. [d] 1, Xxx = Gly; Lys[9] (ϵ-Z); 2, Xxx = Phe; Lys[9] (ϵ-Z); 3, Xxx = Phe; Lys[9] (ϵ-NH_2).

this problem in conjunction with homonuclear correlation spectroscopy (SECSY)[54] or by selective difference decoupling since the chemical shifts of the Lys and Pro protons can be assigned.

Another important feature of the heteronuclear shift correlation is the differentiation between Phe and Trp signals. In this case the carbon β signals are easily assigned due to their distinct chemical shifts (Phe-β, 35–40 ppm; Trp-β, 25–28 ppm). The corresponding proton signals of both amino acid residues are in the typical range around 3 ppm. Hence in the 2D shift-correlation spectrum (Figure 2) the Phe-β and Trp-β correlation peaks are characteristic. If there is at least one β-proton signal of each aromatic amino acid separated in the ^{1}H NMR spectrum from the rest, the *J*-coupling to the α-protons can be used to assign the α signals as well. Homonuclear correlation spectroscopy (or selective decoupling) can be used for this purpose. This result can, however, be obtained in a more direct way by using relayed spectroscopy, which is, in contrast to classical heteronuclear shift correlation, also applicable when β-protons overlap. The relayed spectrum of **1** (Figure 3) indicates strong cross peaks for both α,β-proton couplings in Trp but only for the high-field β-proton of Phe. The missing peak might be retrieved with a different choice for the mixing time τ_m. The relayed experiment together with the evaluation of the homonuclear correlation spectrum and NOE difference spectra, which are used to identify the amino acid sequence[2,55,56] (Table II), yield a complete assignment of the signals in the conformer of **1**.

The same homo- and heteronuclear one- and two-dimensional experiments have been used to assign the spectrum of compound **2** (Tables I and II). The conventional 2D ^{1}H–^{13}C-shift correlation allows assignment of the Pro β- and γ-signals (β, 30.5; γ, 21.1 ppm) (Figure 4). This is in agreement with Veber's findings in the deprotected peptide *cyclo*[Phe-D-Trp-Lys-Thr-Phe-Pro] (**3**) in D_2O (β, 31.6; γ, 22.3 ppm)[7] and proves a cis Phe–Pro bond. The assignment of the carbon signals for **2** was not possible by traditional methods. The overlap of α- and β-proton signals does not allow the assignment of Phe and Trp residues by 2D ^{1}H–^{13}C shift correlation because some proton signals severely overlap (β-protons of Phe[7], Phe[11]; α-protons of Phe[7], Trp[8]). Relayed spectroscopy can solve the assignment problem (Figure 5). The chemical shift difference of the Phe α-proton signals is used to assign the Phe β-carbons. Differentiation between the α signals of Phe[7] and Trp[8] was achieved via the distinct NH chemical shifts of the corresponding amino acids. These proton assignments follow unequivocally from the NOE effects. It is obvious from these examples that the advantage of heteronuclear relayed spectroscopy results from the fact that overlapping proton signals are spread by the carbon chemical shifts in a second frequency dimension (ω_2). The relayed spectrum in Figure 5 exhibits almost all expected correlation peaks; only two signals (marked with circles) are missing in the contour plot. In analogy to conventional 2D ^{1}H-^{13}C shift correlation, the sensitivity decreases with increasing complexity of proton multiplets (e.g., Pro-β,γ,δ; Lys-β,γ,δ).

Table II. NOE Effects of the Cyclic Hexapeptides 1a and 2[a]

	observed NOE effects	
irradiated signal	1	2
NH D-Trp[8]	C_αH Phe[7], 2%	C_αH Phe[7], 12.4%
NH Lys[9]	C_αH D-Trp[8], 6.2%	C_αH D-Trp[8], 7.9% NH Thr, 3.2%
NH Thr[10]	*b*	*b*
NH Xxx[11]	C_αH Thr[10], 3.8%	C_αH Thr[10], 6.1%
NH Phe[7]	no effect	*b*
C_αH Pro[6]	no effect on Phe signals[c]	NH Phe[7], 4.8% C_αH Phe[11], 12%
C_αH Phe[7]	no effect	*d*
C_αH D-Trp[8]	NH Lys[9], 3.5%	*d*
C_αH Lys[9]	C_αH Thr, 4.2%[e]	no effect
C_αH Thr[10]	NH Gly, 0.8%	NH Phe[11], 2.5% C_αH Pro, 4.5%[e]
C_αH Xxx[11]	C_αH Thr, 5.0%[e,f]	C_αH Pro, 8.4%

[a] Obtained by NOE difference spectroscopy at room temperature in Me_2SO. NOE's between protons within an amino acid residue are not indicated. [b] NH signals are covered completely by aromatic resonances. [c] The C_αH signals of Pro, Lys, and Gly (low-field signal) are partially overlapping at room temperature leading to difficulties in selectivity of irradiation. [d] C_αH signals of Phe[7] and D-Trp[8] are completely overlapping from 23 to 90 °C. [e] This effect is in disagreement with all proposed models. A possible explanation could be a minor conformation with a trans Phe–Pro bond. Saturation transfer then could simulate a NOE effect when the chemical shift of $C_\alpha H$ in the trans conformation is identical with that of $C_\alpha H$ Thr. This point needs further clarification. [f] Only the high-field signal was irradiated (3.77 ppm).

Table III. Chemical Shifts of the Aliphatic Carbon Signals in 1a and 2

1a		2	
ppm	signal	ppm	signal
65.77	C_β Thr	66.74	C_β Thr
65.23	Z	65.10	Z
60.85	C_α Pro	60.66	C_α Pro
57.56	C_α Thr	55.70	C_α Thr
55.10	C_α Trp	53.91	C_α Lys
54.61	C_α Lys	53.66	C_α Phe[7]
53.10	C_α Phe	53.16	C_α Trp
46.22	C_δ Pro	52.98	C_α Phe[11]
42.28	C_α Gly	45.81	C_δ Pro
40.17	C_ϵ Lys	39.99	C_ϵ Lys
36.66	C_β Phe	38.22	C_β Phe[7]
30.94	C_β Lys	36.79	C_β Phe[11]
29.03	C_β Pro	30.47	C_β Pro
	C_δ Lys	30.28	C_β Lys
28.88	C_β Trp	28.86	C_δ Lys
23.99	C_γ Pro	27.25	C_β Trp
22.52	C_γ Lys	22.26	C_γ Lys
19.94	C_γ Thr	21.11	C_γ Pro
		17.67	C_γ Thr

Backbone Conformation of Cyclic Hexapeptides

The usual interpretation of the NMR parameters[2] shown above indicates for **2** a conformation with two internal hydrogen bonds, (Phe[7]–CO←HN–Thr[10] and Thr[10]–CO←HN–Phe[7]) similar to the

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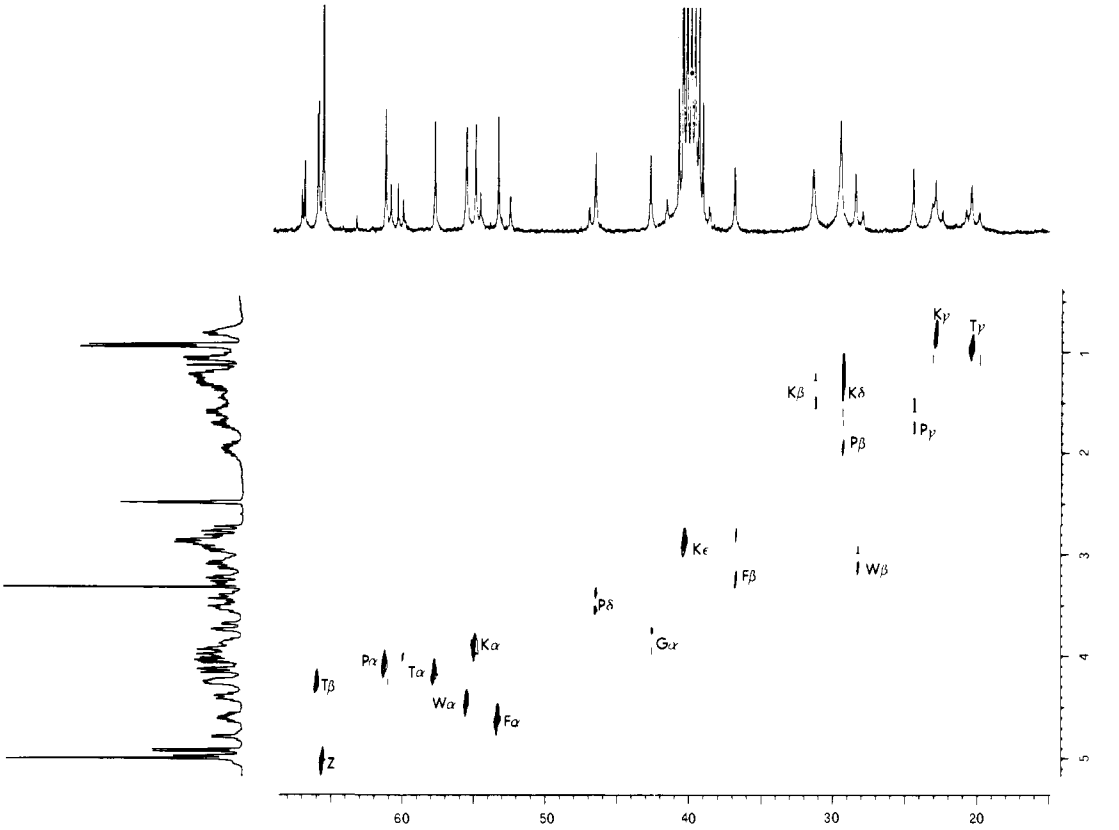

Figure 2. 270-MHz $^{1}H-^{13}C$ shift correlation of *cyclo*[Phe-D-Trp-Lys(Z)-Thr-Gly-Pro] (aliphatic regions in ^{1}H and ^{13}C domain).

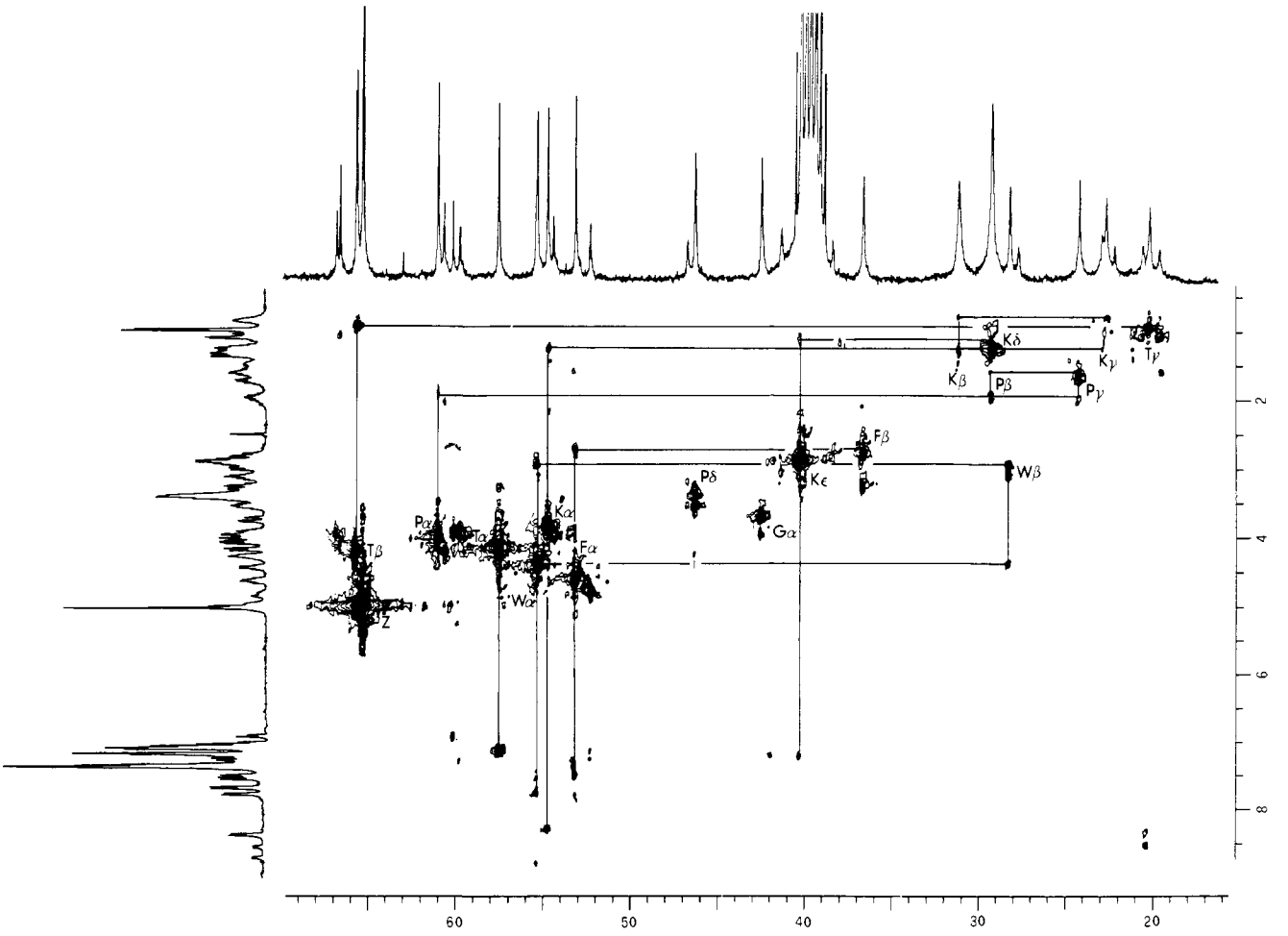

Figure 3. Relayed 300-MHz $^{1}H-^{13}C$ shift correlation of *cyclo*[Phe-D-Trp-Lys(Z)-Thr-Gly-Pro] (full range in ^{1}H, aliphatic region in ^{13}C domain).

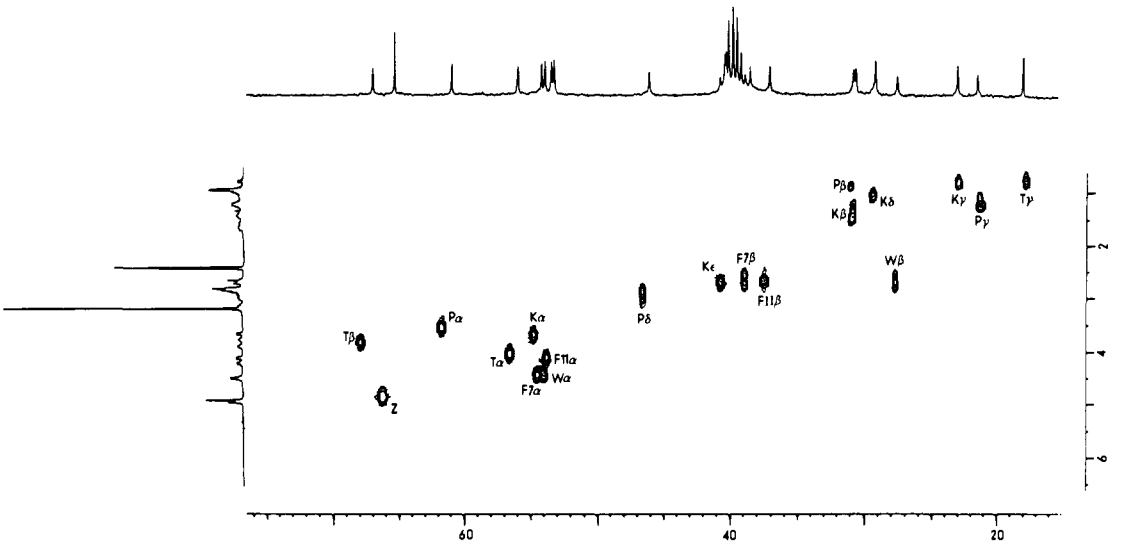

Figure 4. 300-MHz ^1H–^{13}C shift correlation of *cyclo*[Phe^7-D-Trp-Lys(Z)-Thr-Phe^{11}-Pro] (aliphatic regions in both ^1H and ^{13}C domain).

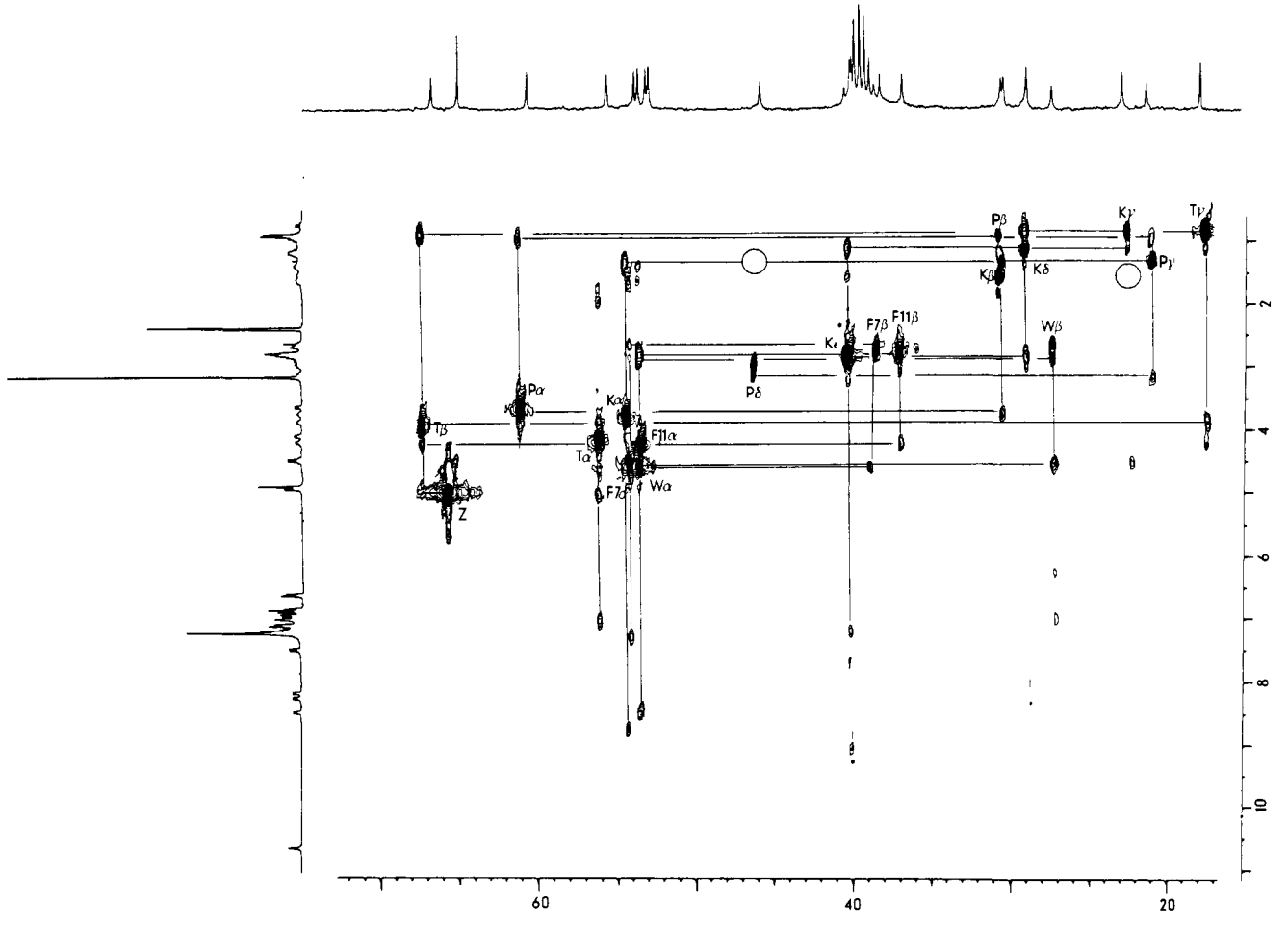

Figure 5. Relayed 300-MHz ^1H–^{13}C shift correlation of *cyclo*[Phe^7-Trp-Lys(Z)-Thr-Phe^{11}-Pro] (full range in ^1H, aliphatic region in ^{13}C domain.)

one proposed for the deprotected compound **3**.[7] The Phe–Pro bonds of both peptides have cis configuration. The strong NOE between α-protons of Phe^{11} and Pro is indicative of their proximity in the β-turn type VI.[57] The NOE from the Lys–NH to Thr–NH and to the α-proton of Trp is important for the identification of the second β-turn, which appears to be of type II′. This is in agreement with expectation from the particular amino acid sequence (D-amino acid in position $i + 1$, L-amino acid in $i + 2$). The evaluation of the coupling constants $^3J_{HNC_\alpha H}$ following the usual Karplus functions for peptides[83] (Table IV) is in moderate agreement with a βII′, βVI conformation (Figure 6). The main discrepancy is observed in the coupling constant of D-Trp, which should be smaller in the case of a βII′ turn. Summing up all arguments we still prefer the conformation of Figure 6.

The similar temperature dependence of the NH chemical shift of the ϵ-Lys-protected peptide **2** in Me_2SO and the unprotected peptide **3** in D_2O indicate a similar conformation of both peptide backbones. The relative stability of the backbone of **2** is further established by results of the solvent titration ($Me_2SO \rightarrow CDCl_3$). Above a concentration of 50 vol % $CDCl_3$ significant high-field

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Table IV. ϕ Angles and $^3J_{HNC_\alpha H}$ Coupling Constants in 1a and 2

1a				2			
amino acid	$^3J_{corr}$,[a] Hz	ϕ,[b] deg	ϕ_{model}, deg	amino acid	$^3J_{corr}$,[a] Hz	ϕ,[b] deg	ϕ_{model}, deg
D-Trp	6.79 ± 0.18	+85/+76		D-Trp	8.27	−36/−87	−20
		−24/−37				+86/+96	
		−80/−93				+144/+157	
		+166/+156	+165 ± 20[c]				
Lys	8.37	−95/−85		Lys	7.22	+29/+42	
		38/80	+55			+76/+91	+90
		−158/−141				−79/−89	
						−151/−164	
Thr	9.60	−105/−90		Thr	7.52	+32/+45	
		+63/+58				+71/+89	
		−140/−135	−125			−80/−90	
						−149/−162	−170
Gly	4.68[d]	+76/+64		Phe[11]	5.99	+21/+33	
	(*pro*-S)	−11/−21				+87/+98	+40
		−96/−105				−71/−81	
		+178/+168	±180			−159/−169	
	4.42[d]	−83/−63					
	(*pro*-R)	+8/+19					
		+97/+108					
		±180/−170	±180				
Phe	9.28	−103/−93		Phe[7]	6.38	+20/+32	
		+48/+68				+85/+95	
		−152/−149	−140			−73/−84	
						−156/−167	−165

[a] Corrected value to consider electronegativity effects.[83] [b] Allowed range of ϕ angle in agreement with the J values. [c] Any β turn with D-Trp in $i+1$ requires $\phi = 60°$ or $-60°$, which is not in satisfactory agreement with the observed value. [d] The low-field proton was assigned to *pro-S* proton due to the anisotropy effect of Thr C=O group.

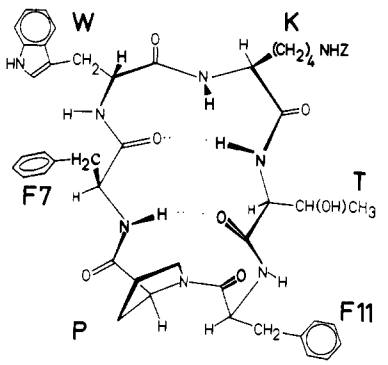

Figure 6. Conformation of **2** in Me_2SO. The numbering of the amino acids follows those of the natural somatostatin.

shifts occur for the externally oriented NH protons (Lys, Phe[11], D-Trp), whereas the internally oriented NH protons (Phe[7], Thr) shift downfield. Some differences in the coupling constants of **2** and **3** are apparent (Table I). The values obtained from Veber[7] for **3** in D_2O correspond better to the expected range for the βII′ turn; however we think that the change of solvent (Me_2SO–D_2O) will influence not only the side chain but also the backbone to a certain degree.

The occurrence of proline in the $i+2$ position of a β turn needs some comment. There are several examples of peptides that involve proline in this position.[58] In all cases in which the preceding amino acid in $i+1$ position has L configuration, the peptide bond between these residues is cis. This is also the case in **2** and again in the cyclic pentapeptide *cyclo*[Phe-D-Trp-Lys-Thr-Pro].[16] A substitution of the L-Thr in the latter peptide by D-Thr changes completely the conformation of the cyclic pentapeptide.[16]

Similarily, a substitution of Phe[11] by the Gly residue in the peptide **1** results in a dramatic change of conformation. In equilibrium a conformation containing a trans Gly–Pro bond dominates (**1a**). The minor conformer (**1b**) probably adopts a conformation similar to **2** and **3**. One indication are the chemical shift values of the NH signals of Trp (8.72 ppm in Me_2SO at room temperature) and Lys (8.49 ppm), which appear at lowest field. These signals are easily assigned by the observation of saturation transfer from the corresponding signal in the major isomer. The carbon NMR spectrum shows small signals for **1b** in the range of β- and γ-carbons of cis Xxx–Pro bonds, which are not yet assigned. The barrier of the interconversion, estimated from the coalescence phenomenon around 100 °C to be about 18–20 kcal/mol, is typical for cis/trans isomerization processes of peptide bonds.[50,51]

In the major conformation **1a** two NH-resonance shifts of adjacent amino acids (Thr, Gly) are not shifted with increasing temperature (Table I), which is considered typical for internal orientation. Furthermore the temperature gradient $\Delta\delta/\Delta T$ of Phe[7]NH is relatively small, pointing to solvent shielding of the NH proton. The present concepts of cyclic hexapeptides[59–68] involve two β turns in a pseudosymmetric arrangement. In contrast to such a possibility, the internal orientation of the Gly–NH in **1a** prevents the adjacent CO group of Thr from being involved in a hydrogen bond as is required for the second β turn. Hence, if Phe[7]–NH is involved in an internal hydrogen bridge, only the Gly–CO is present as proton acceptor (forming a γ turn about Pro). Similar arguments have been used to establish the γ turn in cyclic pentapeptides,[14,69–72] which are now considered as the

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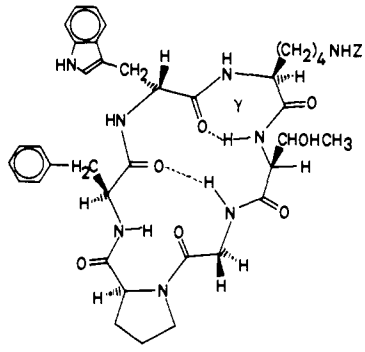

1a

Figure 7. Conformation of **1a** in Me_2SO.

most often occurring turn in cyclic pentapeptides.[2] On the other hand, the $\Delta\delta/\Delta T$ value of 2.9 for Phe[7] is on the upper end of the range for internal orientation. Sometimes such values are found also for external NH orientation, when the group is sterically shielded from the solvent[73] (see also the relatively low value of 2.8 for Phe[11] in **2**). A proline involved in γ turn exhibits an unusual upfield shift of the β-carbon.[72,74] Such an effect is absent in **1a**. We therefore do not assume a γ turn about Pro in the trans-conformation of **1**. Two reasonable alternatives still exist for the two internal NH groups of adjacent amino acids (Thr, Gly). Model consideration clearly shows that Gly–NH can only bind to the same carbonyl (Phe[7]) as Thr–NH if the latter is involved in a β turn similar to the conformation found in the biological active peptide **2**. Such a double-bridged carbonyl group has recently been postulated in other peptides (see, e.g., ref 68), but unequivocal proof still is lacking. The second alternative is a γ turn from (Trp–CO←HN–Thr) but a 1 ← 5 bend of the second NH (Phe–CO←HN–Gly). The evaluation of the coupling constants excludes a β turn about Phe[7]-Trp[8]-Lys[9]-Thr[10] whereas the combination of a 1←3 and a 1←5 bend (Figure 7) is allowed (Table IV).

Note Added in Proof (October 1983): Nitrogen-15 chemical shifts assigned via ^1H-^{15}N shift correlation[14] strongly support the conformation shown in Figure 7. The signals of the nitrogen atoms (Lys, D-Trp) attached to those carbonyl groups involved in hydrogen bonds are clearly shifted downfield from the others.

One point needs a comment. The preference of a β,β-type conformations for cyclic hexapeptides probably is due to the fact that mainly symmetric peptides of the constitution *cyclo*[Xxx-Yyy-Zzz]$_2$ have been investigated. The rapid equilibration of different conformations within the NMR time scale certainly overemphasizes C_2-symmetric conformations. It is therefore reasonable to assume that other conformations probably might often participate or even dominate the conformational equilibrium in cyclic hexapeptides. Our results of five cyclic hexapeptides containing the general structure *cyclo*[D,L-Phe-Phe-D,L-Trp-Lys-Thr-D,L-Phe][46] underline this assumption.

Detailed NMR investigations of somatostatin itself have proven that the natural product has a high degree of flexibility in solution.[75-77] On binding to its receptor the active conformation is picked out by the induced fit.[2] Conformational studies of flexible natural products therefore are of limited value to determine the conformation at the receptor side. On the other hand conformational studies of more rigid derivatives as described above may contribute further to the understanding of conformation and biological activity.[2]

Table V. Schedule for Solid-Phase Synthesis

step	reagent and solvent[a]	times of repetition	time, min
→ 1	CH_2Cl_2	1	3
2	10% TFA, 1% MSA in CH_2Cl_2	1	30
3	dioxane/CH_2Cl_2 1:1	3	3
4	CH_3OH/CH_2Cl_2 1:1	1	3
5	CH_2Cl_2	3	3
6	diisopropylethylamine (DIPEA) 10% in CH_2Cl_2	3	5
7	CH_3OH/CH_2Cl_2 1:1	1	3
8	CH_2Cl_2	3–5	3
9	Boc-amino acid, DCC, HOBT 1:1:1[b]	1	2–4 h
10	CH_2Cl_2	1	3
11	CH_3OH/CH_2Cl_2 1:1	3–5	3
↓			
	or cleavage: 50-fold excess of anhydrous N_2H_4 in DMF, 48 h, room temperature.		

[a] For 1 g of the resin 10 mL of solvent were used usually. [b] 3-fold excess over the estimated first attached amino acid.

Experimental Section

Measurement Conditions. The one-dimensional spectra and the 2D ^1H–^{13}C shift correlation of **1** were measured with a WH 270 Bruker spectrometer. The relayed heteronuclear shift correlation and the shift correlation of **2** were performed on a NT 300 WB Nicolet spectrometer.

^1H–^{13}C Shift Correlation. The applied pulse sequence was ($\pi/2$, ^1H)–($t_1/2$)–(π, ^{13}C)–($t_1/2$)–Δ_1($\pi/2$, ^1H; $\pi/2$, ^{13}C)–Δ_2–acquisition with Δ_1 = 4 ms and Δ_2 = 2.66 ms. All pulses were phase cycled according to ref 34. The spectral width in F_1 was 1500 Hz and in F_2 5000 Hz. The $\pi/2$ pulse was 15 μs for ^{13}C and 47 μs for the ^1H decoupler. A shifted sine bell function was used before Fourier transformation. Zero filling in F_1 was applied. A 90-mg sample of **1** in 5-mm tubes was measured. Total acquisition time was 14.6 h. Spectral width for **2** (at 300 MHz), F_1, 3205 Hz; F_2, 9434 Hz. The $\pi/2$ pulse was 28 μs for ^{13}C and 51 μs for the ^1H decoupler. A 500-mg sample of **1** in 2.5 mL of Me_2SO was measured in a 12-mm tube. Total acquisition time 3.5 h.

Relayed Heteronuclear Shift Correlation. The pulse sequence (Figure 1d) is described above. The same measurement conditions were used as for the 2D ^1H–^{13}C shift correlation for **2** at 300 MHz. Spectral widths: for **1**, F_1 = 3240 Hz, F_2 = 9804 Hz; for **2**, F_1 = 3205 Hz, F_2 = 9434 Hz. $\pi/2$ pulses: 25.9 μs (**1**); 28 μs (**2**) for ^{13}C and 59 μs (**1**); 51 μs (**2**) for the ^1H decoupler. A 300-mg sample of **1** in 3 mL and 500-mg **2** in 2.5 mL solvent were used in a 12-mm tube. Total acquisition time was 15.4 h (for **1**) and 14.5 h (for **2**). The delay τ_m = 15.0 ms was optimized for a coupling $^3J_{HH}$ = 8.3 Hz.

The hexapeptides **1** and **2** have been synthesized from their linear precursors H-D-Trp[8]-Lys(Z)[9]-Thr[10]-Xxx[11]-Pro[6]-Phe[7]-$NHNH_2$ (**1**, Xxx = Gly; **2**, Xxx = Phe) via the azide method according to Medzihradszky.[78] Cyclization yield ranges from 20 to 50% in average after purification steps. The sequence of the linear precursor was chosen due to tactical reasons concerning the solid-phase peptide synthesis: highest cyclization yields are obtained with a D-amino acid residue in N-terminal position,[79] and it is advisable to introduce Trp as late as possible. Boc-PheOH was esterified to hydroxymethylated resin by the action of 2-(dimethylamino)pyridine (DMAP) and dicyclohexylcarbodiimide within 4 h; the unreacted sites of the resin were blocked via benzoylation.[80] After deprotection (10% trifluoroacetic acid (TFA), 1% methanesulfonic acid (MSA) in CH_2Cl_2) couplings were achieved with 3-fold excess of Boc-amino acid, DCC, and 1-hydroxybenzotriazole (HOBT)[81] (Table V).

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Completion of each coupling was checked by a chloranil color test.[82] Threonine was used without benzyl ether protection; possible branching peptides (Boc-D-Trp-Lys-$NHNH_2$) have been eliminated by purification on Sephadex LH20 after cleavage from the resin via hydrazinolysis. The linear Boc protected hexapeptide hydrazides were shown to be homogenous by TLC chromatography and gave the expected amino acid analysis values and reasonable NMR spectra. After deprotection they were cyclized without further purification in high dilution (10^{-3} mmol L^{-1}). Usual workup and purification on Sephadex LH20 or HPLC gave the desired compounds. Satisfactory amino acid analysis was obtained.

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Kinetics of the Scrambling Reaction between Dimanganese and Dirhenium Decacarbonyl

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Abstract: The scrambling reaction between $Mn_2(CO)_{10}$ and $Re_2(CO)_{10}$ to form $MnRe(CO)_{10}$ proceeds cleanly to a stable equilibrium in decalin at 170–190 °C. Studies of the initial rates show that two paths are followed, the major one being evident over the whole range of temperatures and partial pressures of CO above the solutions. The kinetics suggest that it involves prior aggregation to form $Mn_2Re_2(CO)_{20}$ which undergoes stepwise loss of CO to form the clusters $Mn_2Re_2(CO)_{20-n}$ ($n \leq 4$). All these clusters can be envisaged to be quasitetrahedral and to contain one Mn–Mn and one Re–Re bond together with n Mn–Re bonds and $4 - n$ Mn(μ-CO)Re bonds. When the requisite number of CO ligands have been removed the Mn–Mn and Re–Re bonds are lost, either by CO insertion or by redistribution of the M–M and M(μ-CO)M bonds within the cluster. Further CO insertion leads to $Mn_2Re_2(CO)_{20}$ containing two Mn–Re bonds, and this readily fragments to form $MnRe(CO)_{10}$. At low partial pressures of CO the rate-determining step is the formation of $Mn_2Re_2(CO)_{20}$ or $Mn_2Re_2(CO)_{19}$ and the activation parameters are $\Delta H^{\ddagger} = 22 \pm 3$ kcal mol^{-1} and $\Delta S^{\ddagger} = -20 \pm 6$ cal K^{-1} mol^{-1}. The other path is most evident at 190 °C under small partial pressures of CO, and it appears to involve prior loss of CO from the decacarbonyls before aggregation. The kinetics suggest that scrambling occurs mainly via the tetranuclear intermediate $Mn_2Re_2(CO)_{16}$.

It has recently been shown[1] that the scrambling reaction in eq 1 does not occur in decalin under an atmosphere of carbon monoxide at 130–140 °C and that it occurs only very slowly in the absence of CO. This is clear evidence that at least two of

$$Mn_2(CO)_{10} + Re_2(CO)_{10} \rightleftharpoons 2MnRe(CO)_{10} \quad (1)$$

the complexes involved are not undergoing homolytic fission under these conditions as had been proposed.[2,3] Studies of the isotopic scrambling reaction in eq 2 show that it does not occur in *n*-octane

$$^{185}Re_2(CO)_{10} + {}^{187}Re_2(CO)_{10} \rightleftharpoons 2{}^{185}Re{}^{187}Re(CO)_{10} \quad (2)$$

at 150 °C under CO although it is complete after 14 h under argon.[4] While these results show that CO dissociation must play an essential role in the scrambling process they do not provide any evidence for the detailed mechanism of the reactions. We report here some quite extensive kinetic studies of reaction 1 that provide a clear indication of the probable mechanism.

Experimental Section

$Mn_2(CO)_{10}$ (Strem Chemicals) and $Re_2(CO)_{10}$ (Alpha Inorganics) were used as received. Decalin (Aldrich) was purified by successive distillations under reduced pressure until the absorption maxima at 268 and 274 nm due to tetralin were reduced to a negligible value (see Results section). It was stored over molecular sieves. Reactions were carried out in Schlenk tubes fitted with rubber septum caps and immersed in an oil bath (Lauda Model WB-20) maintained to within ±0.1 °C. Oxygen was removed by multiple freeze–pump–thaw cycles under vacuum, and atmospheres of N_2 (Canox Ltd.), CO (Matheson Canada Ltd.), or CO–N_2 mixtures of known composition (Matheson Canada Ltd.) were maintained above the solutions during the reactions. Samples were removed by means of a syringe fitted with a stainless steel needle. IR spectra of the solutions were measured in cells with KBr windows and path lengths of 1.0 or 0.1 mm as convenient. Pye-Unicam SP3-200 or Perkin-Elmer 337 or 180 spectrophotometers were used. UV–vis spectra were measured with a Unicam SP-800 spectrophotometer.

The formation of $MnRe(CO)_{10}$ was established by reaction of $Re_2(CO)_{10}$ with a large excess of $Mn_2(CO)_{10}$. When the absorption due to $Re_2(CO)_{10}$ at 2070 cm^{-1} had been reduced to a negligible value the unreacted $Mn_2(CO)_{10}$ was removed by decomposing it under O_2 at 130 °C.[2] The product solution, after filtration, showed IR bands in the ranges 1800–2300 and 400–750 cm^{-1} that were identical with those reported for $MnRe(CO)_{10}$.[5] The UV–vis spectra of these solutions showed a maximum at 324 nm.[2] After deoxygenation, reaction with triphenylphosphine at 140 °C proceeded to form $MnRe(CO)_8(PPh_3)_2$ with a rate constant of 2.43×10^{-4} s^{-1} in excellent agreement with values obtained from separately isolated $MnRe(CO)_{10}$.[6]

Kinetics were followed by monitoring the decrease in intensity of the distinctive band due to $Re_2(CO)_{10}$ at 2070 cm^{-1}. The absorption due to this band was shown to obey Beer's law. The reaction was also accompanied by a decrease in the intensity of IR bands due to $Mn_2(CO)_{10}$ and an increase in those attributable to $MnRe(CO)_{10}$. The reaction mixtures attained equilibria after ≥30 min. The equilibrium mixtures were stable for as long as 1 h at 190 °C and equilibrium constants were estimated by measurement of the absorbance due to unreacted $Re_2(CO)_{10}$. It was shown that equilibrium could be approached from both directions. Careful attention was paid to the intensities of the bands due to $Mn_2(CO)_{10}$ and $MnRe(CO)_{10}$ so as to establish that no decomposition had occurred. This was revealed clearly in cases where deoxygenation had not been complete, or where levels of tetralin in the decalin had not been

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