Determination of the ϕ Angle in a Peptide Backbone by NMR Spectroscopy with a Combination of Homonuclear and Heteronuclear Coupling Constants

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SYNOPSIS

The ϕ angle in a cyclic peptide is determined by the combined use of homonuclear and heteronuclear coupling constants. Two of the four coupling constants that define the ϕ angle in a peptide are determined exactly, two qualitatively. Via Karplus-type equations, they are transformed into dihedral angles and a good agreement is found, allowing for a determination of the ϕ angle with a certain range of degrees.

Nuclear magnetic resonance spectroscopy is nowadays an established procedure for the determination of small- and medium-sized peptides or proteins in solution.[1-3] Beside the nuclear Overhauser enhancement (NOE) effects, coupling constants can give valuable information during the structure determination. Probably the most useful coupling constants are three-bond couplings, which can directly be converted into dihedral angles via Karplus-type equations.[4-6] They can also be used for obtaining stereospecific assignment of diastereotopic groups that in turn can be used for more accurate utilization of NOE information.[7]

However, while the extraction of distance information from NOE effects has been extensively used and is quite straightforward, the information in principle available from coupling constants has only been used in few cases. The reason for that is twofold: The extraction of coupling constants from nmr spectra with reasonable accuracy is not as straightforward as with the NOE information, especially with larger molecules, where the line width is in the range of the coupling constants or even larger. The conversion of the value for the coupling constant into a bond angle is not unambiguous, since usually four dihedral angles correspond to one coupling constant in a typical Karplus-type equation.

The latter problem can be overcome with the combined use of several coupling constants defining the same dihedral angle.[31] These will usually include heteronuclear coupling constants, and thus one needs sensitive methods for the determination of coupling constants that are line width independent.

One important concept to determine coupling constants independent from the line width is the creation of multiplet patterns with selection of only connected or nonconnected transitions [i.e., exclusive correlated spectroscopy (E.COSY) type patterns], from which the coupling constant is extracted by measuring the displacement of basic patterns. If homonuclear coupling constants have to be determined, the original E.COSY procedure[8-10] works with a suitable combination of COSY spectra with multiple quantum filter of different orders to select either connected or nonconnected transitions.

Recently, Wagner's group has demonstrated that heteronuclear coupling constants to a proton-bearing heteronucleus can accurately be measured from homonuclear spectra [either total (TOCSY) or NOE spectroscopy] of isotopically enriched proteins.[11] This relies on the fact that the heterospin, which is not pulsed during a homonuclear experiment, does not change its spin state and only connected transitions can 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 hetero-

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nuclear long-range coupling is visible as a displacement of two peaks, which are separated by a large heteronuclear one-bound coupling, and can thus be measured with the usual high intensity of a homonuclear spectrum, independent from the line width. Another advantage is that the possibility of measuring the heteronuclear coupling constant is not dependent on the size of the coupling constant, but relies on other transfer processes (homonuclear coupling constants or NOE effects). Here we show that in case of smaller molecules, where an isotopic enrichment is usually not feasible, the same effect can be achieved by implementing a proper half-filter[12] in the homonuclear technique. An extension of this sequence to a three-dimensional experiment is then straightforward.[13-15] In case of a protein the heavy overlap of resonances will also make this extension to a higher dimension necessary.

There is, however, one drawback with this method. The proton bound to the heteronucleus is of crucial importance since it provides the large heteronuclear coupling constant, which provides the independence from the line width and guarantees the high sensitivity. Thus coupling constants to carbonyl carbons cannot be determined and one has to resort to the HMBC[16] experiment. It has been shown that coupling constants can be measured with high accuracy from HMBC spectra with some computational effort.[17,18] This, however, always requires the presence of the peak in the spectrum, which is difficult to obtain if the coupling constant is small. But then a qualitative analysis is still possible, which may be sufficient to resolve remaining ambiguities. To get this qualitative information on coupling constants to carbonyl carbons, a selective HMBC[19,20] and a comparison of cross-peak intensities, which are dependent on the size of the active heteronuclear coupling constant, is probably the best method.

We want to demonstrate the combined use of homonuclear and heteronuclear coupling constants for the determination of the ϕ angles of the cyclic hexapeptide *cyclo*[-D-Pro-Phe-Phe-Lys(Z)-Trp-Phe-] (F3), an analogue of the potent inhibitor of a hepatic transport system, 008.[21] Only Thr[3] has been substituted by a phenylalanine.[22]

It can be seen from Figure 1 that the ϕ angle in a peptide can be determined by four three-bond coupling constants from protons to either other protons or heteronuclei: ${}^3J[H^{\alpha}_{(i)}\text{-}H^{N}_{(i)}]$, ${}^3J(H^{N}_{(i)}\text{-}C^{\beta}_{(i)}]$, ${}^3J[H^{N}_{(i)}\text{-}C'_{(i)}]$, and ${}^3J[H^{\alpha}_{(i)}\text{-}C'_{(i-1)}]$. The relevant Karplus-type equations are as follows[6]:

$$^3J(H^N\text{-}H^\alpha) = 9.4\cdot\cos^2(\phi - 60) - 1.1\cdot\cos(\phi - 60) + 0.4$$

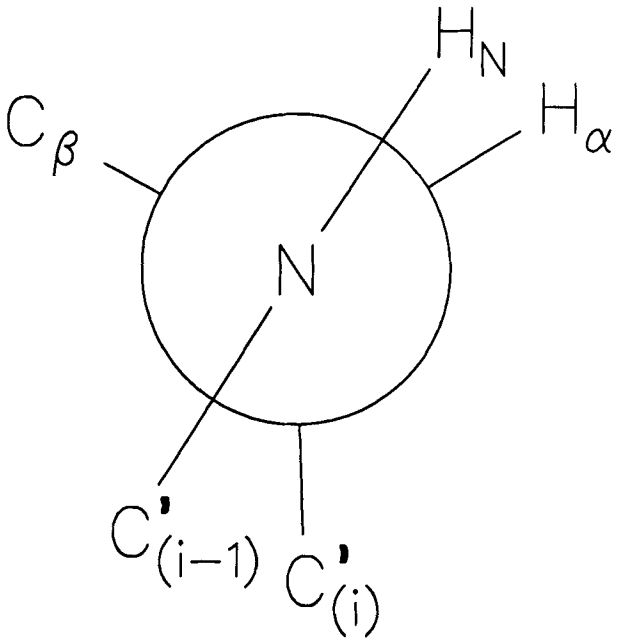

Figure 1. Newman projection along the N-C$^{\alpha}$ bond of a peptide backbone. It can be seen that four proton–proton or proton–carbon coupling constants can be utilized to determine the ϕ angle. According to the IUPAC nomenclature, ϕ is measured between the two carbonyl carbons.

$$^3J(H^N\text{-}C^\beta) = 4.7\cdot\cos^2(\phi + 60) - 1.5\cdot\cos(\phi + 60) - 0.2$$

$$^3J(H^N\text{-}C'_{(i)}) = 5.7\cdot\cos^2(\phi - 180) - 2.7\cdot\cos(\phi - 180) + 0.1$$

$$^3J(H^\alpha\text{-}C'_{(i-1)}) = 9.0\cdot\cos^2(\phi + 120) - 4.4\cdot\cos(\phi + 120) - 0.8.$$

where ϕ is the angle defined by the two carbonyl carbons, according to the IUPAC nomenclature. A graphical representation of the four equations is given in Figure 2. The homonuclear coupling constant can be determined very easily within a cyclic hexapeptide from the one-dimensional spectrum or the double quantum filtered COSY spectrum. In a protein a more elaborate scheme for the determination of this important coupling constant might be necessary and several methods have been proposed in the literature.[23-26]

The region of the H^N protons in the one-dimensional spectrum of F3 is shown in Figure 4a, and the values of the coupling constants are given in Table I. The heteronuclear ${}^3J(H^N\text{-}C^\beta)$ can be determined from the ${}^{13}C\text{-}\omega_1$ half-filtered TOCSY experiment (HETLOC, determination of *HET*eronuclear *LO*ng-range *C*ouplings[27]).

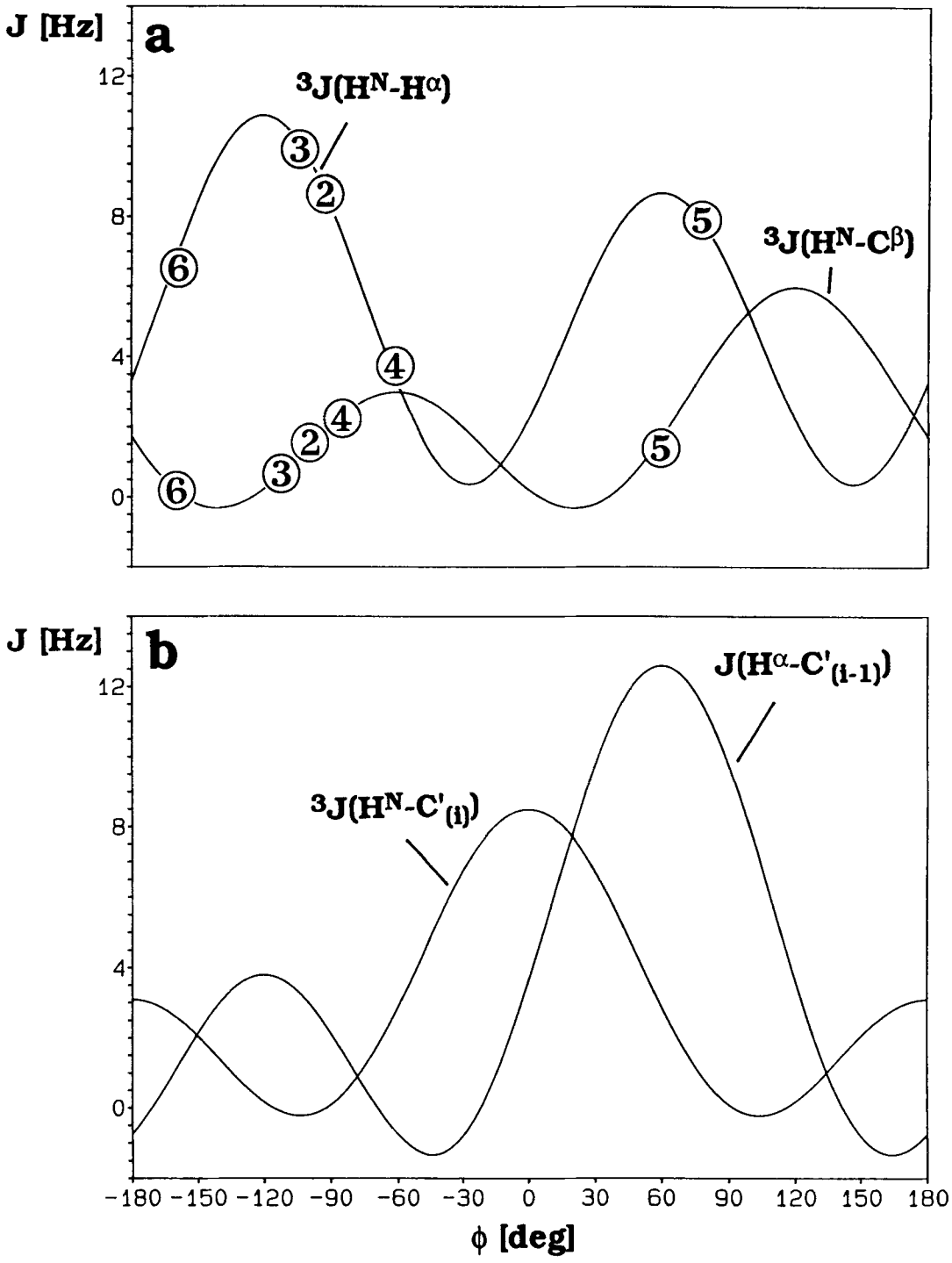

Figure 2. Graphical representation of the four Karplus equations given in the text. (a) Equations of those coupling constants that were measured exactly. The numbers indicate the five amino acid residues and the dihedral angle corresponding to the coupling constant. Only those values are given that remain after exclusion based on all available coupling constants. (b) Equations of the two coupling constants to carbonyl carbons. Here coupling constants were only estimated from the intensity of the peaks. Coupling constants below 1 Hz did not yield a correlation in the HMBC.

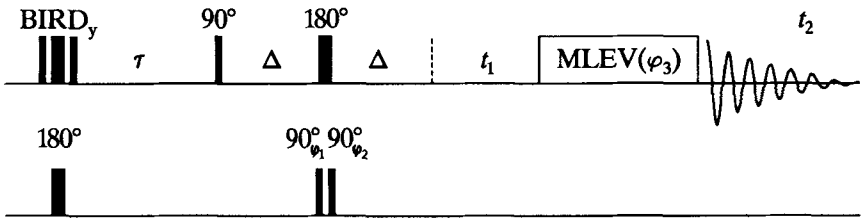

Figure 3. Pulse sequence of the HETLOC experiment. The delay Δ is $({}^1J_{HX})^{-1}$, the phase cycle is $\phi_1 = x, -x, x, -x$, $\phi_2 = x, x, -x, -x$, $\phi_3 = x, x, x, x, -x, -x, -x, -x$, rec. = +, −, −, +. The BIRD pulse[29] is used for the presaturation of the protons bound to ^{12}C and allows for rapid pulsing if used according to Bax and Subramanian.[30]

The pulse sequence is given in Figure 3. A BIRD pulse[29] is applied in front of the sequence, which results in a very good suppression of protons bound to ^{12}C, and allows for rapid pulsing with two scans per second if executed according to the method of Bax and Subramanian.[30] The sequence starts with a ω_1 half filter, and the delay Δ is set to $(2^1J_{HC})^{-1}$. The two heteronuclear 90° pulses are phase cycled in the usual way[12] to result in an alternation of an 180° pulse and a 0° pulse. At the end of the two delays in-phase proton magnetization is present with negligible phase distortions from homonuclear proton–proton couplings. The following part of the sequence is then the well-known TOCSY sequence, which is now executed with protons bound to ^{13}C only. Thus a splitting of the signals due to the large heteronuclear one-bond coupling will appear in the F_1 dimension. The TOCSY mixing sequence will transfer this magnetization to protons bound to ^{12}C, which will exhibit a small heteronuclear long-range coupling to the same carbon nucleus, which caused the splitting in the F_1 dimension, in the F_2 dimension. The result is a cross peak with the desired E.COSY type pattern.

The spectrum obtained with this sequence is shown in Figure 3b and the coupling constants determined from the E.COSY type patterns are given in Table I. After determining dihedral angles from the two types of coupling constants found so far, there is still some ambiguity, which can be resolved from a qualitative inspection of a selective HMBC. The region of the H^N-C′ signals is shown in Figure 4c. The spectrum has been recorded with a delay of 80 ms for developing the heteronuclear coupling constant. Thus the absence of a peak indicates that the heteronuclear coupling constants is smaller than one hertz. This can be estimated from an HMBC recorded with the full carbon spectrum and the same delay from peaks resulting from known coupling constants. Here only three H^N-C^β peaks are visible, those from F^2, K^4, and W^5. It can be seen from Figure 3c that only F^6 shows a correlation to its own carbonyl carbon. Additional information can be obtained from the H^α-$C'_{(i-1)}$ region of the same spectrum (data not shown). The correlations of F^3 and F^6 are of medium size, the correlation of W^5 is very strong, other correlations are not visible because of overlap, but obviously sufficient information is given by the other three coupling constants. While following that procedure, it should be kept in mind that the intensities of the cross peaks are also influenced by the homonuclear coupling constants.[19] Thus only

Table I Coupling Constants and Corresponding Angles for the Cyclic Hexapeptide *cyclo*-D-Pro-Phe-Phe-Lys(Z)-Trp-Phe-[a]

	$^3J(H^N\text{-}H^\alpha)$	Angle from $^3J(H^N\text{-}H^\alpha)$	$^3J(H^N\text{-}C^\beta)$	Angle from $^3J(H^N\text{-}C^\beta)$
Phe-2	8.6	66.1, 53.9, −148.7, **−91.3**	1.3	−18.3, **−101.7,** 55.3, −175.3
Phe-3	9.6	−141.2, **−98.8**	0.5	−5.3, **−114.7,** 44.9, −164.9
Lys-4	3.3	111.9, 8.1, ±180, **−60**	2.0	−24.6, **−95.4,** 62.7, 178.3
Trp-5	8.0	**76.3,** 43.7, −152.6, −87.4	1.5	−21.4, −98.6, **57.6,** −177.6
Phe-6	6.6	89.2, 30.8, **−160.9,** −79.1	0.2	0.5, −120.5, 40.0, **−160.0**

[a] The bold values correspond to those dihedral angles that are in agreement with all four coupling constants.

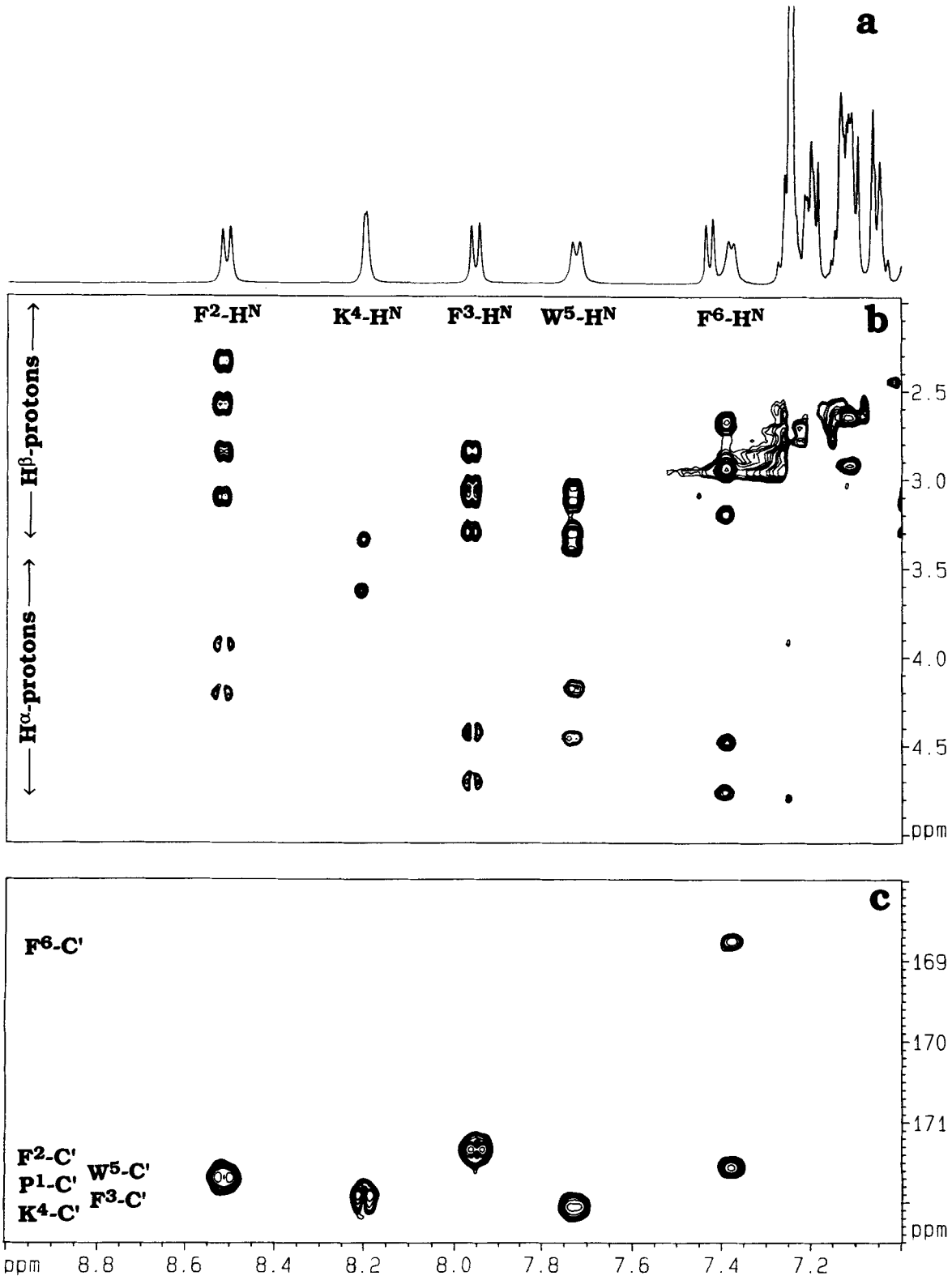

Figure 4. Spectra of the cyclic hexapeptide *cyclo*[-D-Pro^1-Phe^2-Phe^3-Lys(Z)^4-Trp^5-Phe^6]. All spectra were recorded on a Bruker AMX-500. (a) Region of the one-dimensional proton spectrum. (b) Region of the H^N to H^α/H^β peaks in the HETLOC spectrum, the artefacts in the upper right are quadrature artefacts of the aromatic resonances. (c) Region of the H^N to C′ peaks in the selective HMBC. Only the H^N of F^6 shows a correlation to their own C′, indicating that the coupling constant is larger than 1 Hz.

a comparison of peaks resulting from the *same* proton {e.g., peaks resulting from a known two-bond coupling [$^2J(H^{\alpha}_{(i)}\text{-}C'_{(i)})$] and a peak from a three-bond coupling [$^3J(H^{\alpha}_{(i)}\text{-}C'_{(i-1)})$]} is possible without a calculation of the transfer functions.

The ϕ angle can now be determined (bold values in Table I); however, it should be noted that these angles can only be understood as the center of an allowed region. A further step of structure determination has to be the implementation of a suitable pseudo-potential into a molecular dynamics force field, to allow for an appropriate use of the determined dihedral angle as a structural parameter. An example has already been given in the literature.[28]

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