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High-Resolution Solid-State NMR Structure of an Anticancer Agent

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We demonstrate that solid-state NMR methods can be used to rapidly determine the high-resolution 3D structure of Epothilone B in the polycrystalline state. The solid-state NMR structures exhibit an average heavy atom RMSD to the mean structure of 0.14 Å. The 3D-structural analysis leads to stereospecific assignments and provides insight into the influence of intermolecular interactions upon ssNMR chemical-shift parameters. Our results pave the way to the study of ligand–microtubule interactions in a noncrystalline and insoluble environment at atomic level.

Introduction

Solid-state Nuclear Magnetic Resonance (ssNMR) has recently made significant progress in probing molecular structures in applications ranging from solid-phase peptides and small proteins[1–8] to protein fibrils[5,9–13] and membrane protein complexes[14–16]. In addition, the structural characterization of nonpeptidic molecules and the conformational changes associated with their coordination in a (bio)chemical matrix or within the bulk of larger structures is of high technological and pharmacological interest. Classical ssNMR parameters to detect molecular interactions are chemical-shifts and internuclear distances. To discriminate between structural changes and intermolecular interactions, the precise determination of 3D molecular structure must be based on the measurement of a sufficient number of internuclear distances. Whereas dipolar (^{13}C,^{13}C) and (^{13}C,^{15}N) recoupling methods can yield individual distance constraints with high accuracy (for example see references [1, 2, 5 17]), 3D molecular structures of larger polypeptides have been obtained predominantly using (^{13}C,^{13}C) or (^{1}H,^{1}H) mixing schemes for which the polarization transfer dynamics are best described using relaxation theory in a coupled spin network.[4,18,19] For example, proton–proton distances detected indirectly using N/CHHC pulse schemes,[4,20] have been used in the context of small molecules,[21,22] proteins,[6,7,16] and nucleic acids.[23]

Herein, we show for Epothilone B (Figure 1) that two 2D CHHC correlation experiments are sufficient to rapidly assemble a high-resolution structure of nonpeptidic molecules. Epothilones are natural compounds produced by the myxobacterium *Sorangium cellulosum*.[24,25] They are known to stabilize microtubules, the polymerized form of the heterodimeric protein αβ-tubulin.[26] The extensive polymerization of αβ-tubulin induced by epothilones triggers apoptosis (programmed cell death). As epothilones exhibit high cytotoxic activity against multiresistant tumor cells,[25–27] there is a strong interest in these compounds as potential drugs in antitumor therapy.

A detailed understanding of the functional mechanism for the interaction of epothilones with αβ-tubulin would allow for the structural-based design of pharmacologically optimized compounds acting on the tubulin polymerization equilibrium. Whereas the crystal structure of free epothilone B is known,[24] its bioactive conformation is still a matter of debate.[28] The first study of the bioactive conformation of epothilones was conducted by solution-state NMR for epothilone A (EpoA, which lacks the methyl group at C12) bound to nonpolymerized αβ-tubulin.[29] In addition, the tubulin/EpoA complex was studied by electron-crystallography for Zn^{2+}-stabilized αβ-tubulin layers.[30] The conformation of the tubulin-bound epothilone is different in the two studies, suggesting the need for further investigation. As this discrepancy may reflect the dependence of the epothilone binding mode on the tubulin

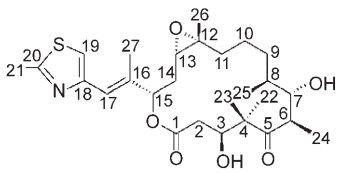

Figure 1. The natural product epothilone B by the myxobacterium *Sorangium cellulosum*.

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polymerization state, it is important to derive the conformation of epothilone bound to the biologically relevant polymerized tubulin, that is, to microtubules (MT). The investigation of the complex of epothilones with MTs is challenging for solution-state NMR spectroscopy and crystallographic techniques. On the other hand, solid-state NMR experiments on labeled epothilone in complex with nonlabeled microtubules could in principle be used to elucidate the tubulin-bound structure of the drug. The strategy presented herein provides the proof that such structural investigations are indeed feasible.

The structure determination approach described in the following relies on the indirect detection of short proton-proton contacts under Magic Angle Spinning (MAS)[31] conditions. The corresponding CHHC[4,6,20] correlations experiments were performed to investigate whether through-space proton–proton distances alone are sufficient to determine the 3D structure of epothilone B. Unlike a similar study on L-tyrosine-ethylester,[21] where intrinsic molecular-dynamic processes are present, we here show that a CHHC-based ssNMR analysis can lead to a high-resolution structure including the stereospecific assignment of all carbon positions of Epothilone B.

Results and Discussion

2D solid-state NMR on Epothilone B. First, a ^{13}C proton-driven spin diffusion[32] spectrum with a mixing time of 10 ms was recorded (Figure 2). At this mixing time, primarily one-bond correlations are detected. The observed ^{13}C line widths are well below 1 ppm indicating that the investigated epothilone B sample consists of nano- or microcrystalline powder. The large dispersion of the resonances (ranging from 15.2 to 222.7 ppm) and the high spectral resolution makes the unambiguous assignment of all 27 ^{13}C resonances (see Table 1) feasible.

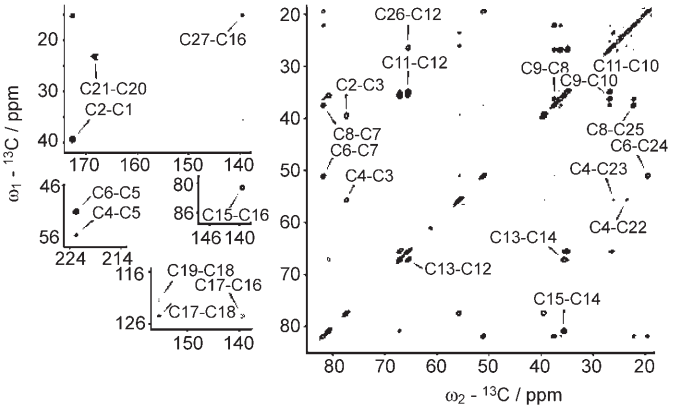

Figure 2. 2D ^{13}C spin-diffusion spectrum (mixing time: 10 ms) of [^{13}C] labeled epothilone B recorded on a 600 MHz NMR spectrometer (Bruker Biospin) at 11.9 kHz MAS. At this mixing time mostly one-bond correlations are observed.

The stereospecific assignment of C22 and C23 was obtained by CHHC correlations. Two CHHC spectra with ^{1}H-^{1}H mixing times of 100 μs (Figure 3) and 150 μs were recorded. As described in detail in reference[4], at these mixing times and at an MAS rate of 11 kHz ($B_0 = 14.1$ T), CHHC spectra are dominated by ^{1}H-^{1}H interactions corresponding to distances of 1.8–3.0 Å. All CHHC cross-peaks are well-resolved and can be assigned unambiguously. As the [^{13}C] labeled epothilone B was diluted in natural abundance material, all CHHC cross-peaks result from intramolecular polarization transfer. CHHC spectra of undiluted small organic molecules contain a large fraction of intermolecular cross-peaks because of their small ratio of volume and surface. Therefore, isotopic dilution is crucial to perform a 3D structure determination based on CHHC correlations. The problem of intermolecular cross-peaks would not be present in the complex of epothilone B with microtubules, where the epothilone B binding pockets are well separated in space.

Structure calculation. To construct the 3D structure of epothilone B, molecular-dynamics based structure calculations in CNS[33] were performed. ^{1}H-^{1}H distance constraints obtained from CHHC 2D spectra were represented by square-well potentials (see Table 2). [1.8, 2.5] Å intervals were used for strong cross-peaks (that is, 100–50% peak volume compared to the strongest cross-peak) in the 100 μs CHHC spectrum. For weak cross-peaks and those appearing only at a mixing time of 150 μs, [1.8, 3.0] Å intervals were used.

As ^{1}H-^{1}H interactions are detected indirectly through the spins of bonded ^{13}C atoms, the assignment of methylene and methyl protons remains ambiguous. Following solution-state

Table 1. ^{13}C chemical shift assignments of Epothilone B in ppm.

spin[a]	Solid-state NMR	Liquid-state NMR[b]	$\Delta\delta = \delta$(solid)-δ(solution)
C1	172.7	174.1	-1.4
C2	39.5	42.8	-3.3
C3	77.5	74.5	3.0
C4	55.7	57.2	-1.5
C5	222.7	221.4	1.3
C6	51.1	48.8	2.3
C7	81.8	79.4	2.4
C8	37.5	39.5	2.0
C9	36.3	33.5	2.8
C10	26.8	27.0	-0.2
C11	34.9	36.1	-1.2
C12	65.6	65.0	0.6
C13	67.1	65.4	1.7
C14	35.6	36.9	-1.3
C15	80.8	80.5	0.3
C16	139.4	141.2	-1.8
C17	124.5	123.1	1.4
C18	155.5	156.0	-0.5
C19	121.4	121.6	-0.2
C20	168.5	168.3	0.2
C21	23.3	22.8	0.5
C22	23.5	23.6	-0.1
C23	26.1	26.4	-0.3
C24	19.5	20.4	-0.9
C25	22.2	22.3	-0.1
C26	26.1	26.0	0.1
C27	15.2	18.1	-2.9

[a] Numbering as in Figure 1. [b] Referenced to the upfield resonance of adamantane at 31.47 ppm in DMSO.

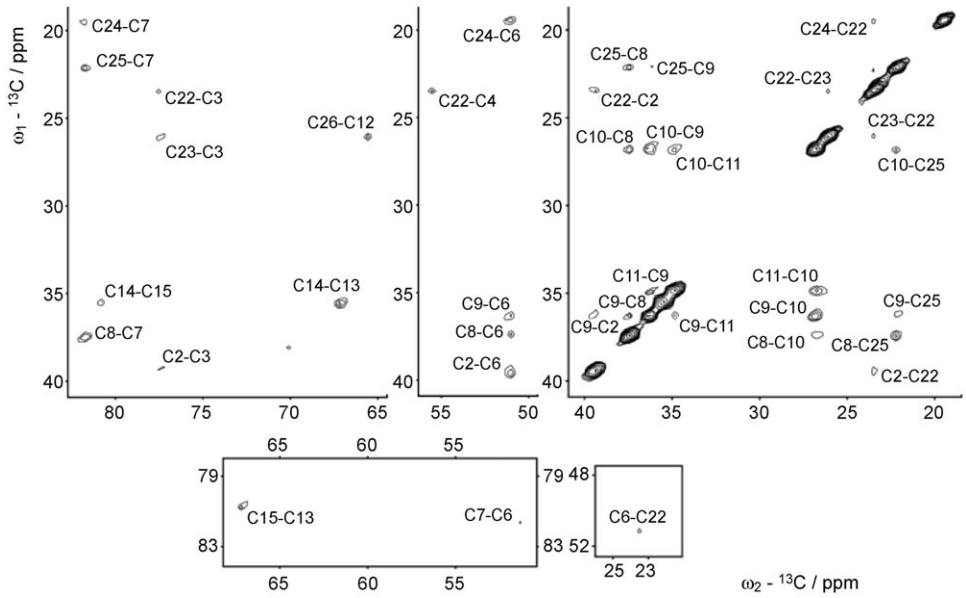

Figure 3. 2D CHHC spectrum (^{1}H-^{1}H mixing time: 100 μs) of [^{13}C] labeled epothilone B recorded on a 600 MHz NMR spectrometer (Bruker Biospin) at 11 kHz MAS. The assigned correlations reflect intramolecular through-space proton–proton connectivities.

Table 2. ^{1}H-^{1}H distance constraints at 100 μs[a] and only present at 150 μs[b] mixing time.

C [#][a]	C [#]	Volume
7	8	100.0
9	11	87.8
9	10	84.5
2	6	79.3
13	15	71.0
10	11	57.7
7	25	55.8
2	22	50.6
6	9	50.6
2	9	42.0
6	24	41.4
8	9	36.7
8	10	36.5
13	14	34.3
3	23	31.8
8	25	28.5
9	25	23.4
7	24	18.7
10	25	18.3
2	3	17.4
6	8	17.0
3	22	15.9
14	15	15.5
6	7	10.6
22	24	10.4
6	22	10.4
22	23	8.9
C [#][b]	C [#]	
15	17	
17	19	
2	8	

NMR spectroscopic approaches,[34] this aspect was accounted for during the calculation by using an r^{-6} summation involving all possible ^{1}H-^{1}H contacts (see also reference [4]). In total, 30 CHHC constraints were used to calculate an ensemble of 200 solid-state NMR structures. The calculations were performed twice for both possible stereospecific assignments of C22 and C23. With the assignment listed in Table 1, the ten lowest energy structures exhibit an average total energy of E_{total} = 46 kcal mol^{-1} and an average contribution from CHHC constraint violations of E_{CHHC} = 0.8 kcal mol^{-1}. This shows that all CHHC constraints can be fulfilled simultaneously. In contrast, the opposite assignment of C22 and C23 leads to values of E_{total} = 61 kcal mol^{-1} and E_{CHHC} = 11 kcal mol^{-1}, demonstrating that it is not possible to fulfill all CHHC constraints simultaneously. As a result the stereospecific assignment as listed in Table 1 follows. This assignment agrees with NOE-based assignments by Höfle et al.[24] but differs from liquid state NMR results obtained using a combination of NOEs, $^3J_{HC}$ and $^3J_{CC}$ scalar couplings.[29]

Figure 4 shows the comparison of the ten lowest energy solid-state NMR structures (in black) and the X-ray structure[24] (in gray). The ten solid-state NMR structures exhibit an average

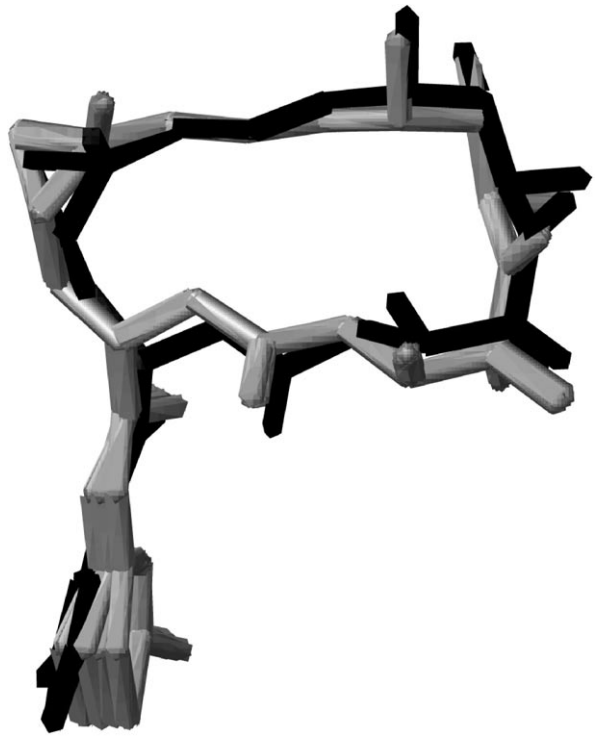

Figure 4. Comparison of the ten lowest energy solid-state NMR structures derived from CHHC data (shown in grey) and the x-ray structure by Höfle et al.[24] (shown in black). Molecules were aligned using MOLMOL 2 K.2.[35]

heavy atom RMSD to the mean structure of 0.14 Å. The average heavy atom RMSD to the X-ray structure is 0.75 Å.

Comparison to other results. When comparing ssNMR assignments with previous solution-state NMR data, significant shift differences (that is, $\Delta\delta = \delta(\text{solid}) - \delta(\text{solution})$) are observed for several carbon positions (Table 1, see also Figure 5). According

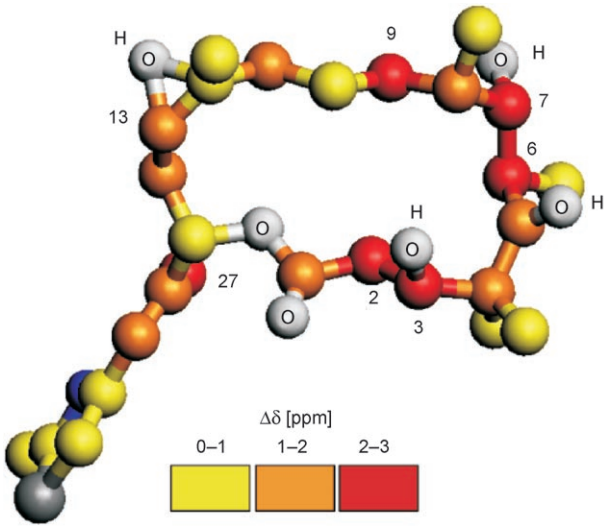

Figure 5. Ball and stick representation of solid-state epothilone B with the following color coding: oxygen atoms: grey with corresponding lettering; nitrogen atoms: blue; sulfur atoms: dark grey; carbon atoms: yellow to red according to chemical-shift perturbation (given in absolute values). Oxygen atoms involved in inter-molecular hydrogen bond formation are indicated with the letter H.

to Höfle et al., the conformation of the macrocycle in solution corresponds to the one found in the crystal. However, there is no preferred conformation of the thiazole side chain. For example, in solution NOE[36] effects for H19-H17 and H19-H27 are observed simultaneously. This indicates a rotational degree of freedom of the side chain around the C17–C18 bond which would explain the observed chemical shift change for C17. In crystals obtained from dichloromethane (space group $P2_1$), intermolecular hydrogen bonds are present between C3-OH and the epoxide oxygen, and between C7-OH and the C5 carbonyl group.[24] Indeed, considerable chemical shift changes at carbon positions CX-OH (X = 1,3,5,7, and 13) are seen, suggesting a qualitative correlation between chemical-shift variations and intermolecular ^{1}H bonding. The absolute size of $\Delta\delta$ and chemical-shift perturbations seen for neighboring carbon positions may be caused by relay effect or by minor conformational changes that are not detected by CHHC or NOE experiments with the necessary accuracy and precision. Moreover, the partial flexibility of the epothilone macrocyclic ring in solution,[37] may lead to a mixture of conformations in solution in contrast to a well defined conformation in the solid state. Finally, differences in the stereospecific assignments in C22 and C23 between solution[29] and solid-state NMR may be due to intermolecular interactions in the polycrystalline sample where the methyl groups at the C4 position are situated below the thiazole ring of a neighboring epothilone molecule producing strong ring current effects. Intermolecular correlations could be further investigated using CHHC data on undiluted samples or by conducting (^{1}H,^{1}H) correlation spectroscopy.[19] The latter experiments would, however, not be applicable for epothilone B/MT complexes.

Conclusions

The 3D structure of solid-phase epothilone B has been determined using a small set of two-dimensional solid-state NMR experiments. One ^{13}C spin diffusion experiment was sufficient to assign 25 out of 27 ^{13}C resonances unambiguously. Two CHHC experiments with mixing times in the initial rate regime yielded 30 ^{1}H-^{1}H distance constraints in the range of 1.8–3.0 Å and led to the stereospecific assignments of C22 and C23. At this mixing time, relay peaks are negligible and do not interfere with the structural analysis. Instead of choosing very short mixing times, relay peaks could also be dealt with by acquiring double-quantum CHHC[4,20] spectra that allow (similar to ROESY[38,39] experiments in solution) a clear classification between direct and relay peaks. Within the accuracy and precision of the experimental CHHC data one can conclude that the structure of epothilone B in a crystal (determined by X-ray crystallography)[24] and as a powder (determined by solid-state NMR) are identical.

To obtain a similarly large constraint set with solid-state NMR spectroscopic methods that rely on the detection of individual ^{13}C–^{13}C distances,[40–42] a build-up of two-dimensional experiments would have to be performed for each distance. Even though the CHHC experiment involves three CP transfer steps and is thus less sensitive than chemical shift selective transfer methods, which involve only one CP, the total time needed to obtain the 3D structure is drastically reduced. The observed chemical shift differences between solid-state and solution-state chemical shifts can be largely explained by the rotational degrees of freedom of the thiazole side chain and partially of the macrolide ring and the formation of intermolecular hydrogen bonds involving oxygen atoms from the macrocycle. This indirectly confirms that the crystallites in the powder-like solid-state NMR sample are identical to the single crystal.[24] The high sensitivity of the solid-state NMR chemical shifts to the formation of hydrogen-bonds could prove useful in the context of epothilone B/microtubule interactions. There, a comparison of chemical shifts in free and microtubule-bound form would allow for a detection of hydrogen-bonds between epothilone-B and polymerized $\alpha\beta$-tubulin.

Experimental Section

Sample preparation. [^{13}C] labeled epothilone was obtained by shake flask fermentation with the myxobacterium *Sorangium cellulosum* So ce90 by using a high-epothilone-producing mutant, BCE99/41 (Novartis strain collection). The fermentation was performed by growing the strain in a medium (1000 mL) containing [^{13}C] labeled starch ($^{13}C_6$, 98%; 20 g) as the carbohydrate source. 20 mg of epothilone A and 15 mg of epothilone B were obtained

with an incorporation rate of 60–70% ^{13}C. 3 mg of [^{13}C] labeled epothilone B and 18 mg of unlabeled epothilone B were dissolved in dichloromethane and recrystallized.

Solid-state NMR experiments. Two-dimensional NMR experiments were conducted on a 14.1 T (^1H resonance frequency: 600 MHz) wide-bore instrument (Bruker Biospin, Germany) equipped with a 4 mm triple-resonance (^1H, ^{13}C, ^{15}N) MAS probe. All experiments were carried out at probe temperatures between $-5\,^{\circ}C$ and $-8\,^{\circ}C$. High power proton-decoupling using the sequences TPPM[43] or SPINAL64[44] with r.f. amplitudes of 80–90 kHz was applied during evolution and detection periods. All spectra were processed using QSINE window functions in F1 and F2 and analyzed using Sparky version 3.111 (T. D. Goddard and D. G. Kneller, University of California). For the assignment of ^{13}C resonances a conventional proton-driven spin diffusion (SD) scheme employing a longitudinal mixing time of 10 ms was used (MAS frequency: 11.9 kHz). The contact time t_{HC} of the ramped cross-polarization was set to 1 ms. For the indirect detection of ^1H-^1H correlations two CHHC spectra with ^1H-^1H mixing times of 100 μs and 150 μs were acquired (MAS frequency: 11 kHz). Short contact times of t_{HC} = 100–150 μs enclosing the ^1H-^1H transfer step ensured polarization transfer within bonded ^1H-^{13}C pairs only. The contact time of the initial cross-polarization was set to 1 ms. ^{13}C resonances were calibrated using adamantane as an external reference. The upfield resonance of adamantane was set to 31.47 ppm to allow for a direct comparison of the solid-state chemical shifts to solution-state NMR data. The assigned solid-state chemical shifts are given in Table 1 (for the numbering of the ^{13}C nuclei see Figure 1). For comparison, Table 1 also contains solution-state chemical shift assignments for epothilone B in DMSO referenced to the upfield shift of adamantane at 31.47 ppm. All detected CHHC cross-peaks could be assigned unambiguously. Cross-peaks from the spectrum with a ^1H-^1H mixing time of 100 μs were integrated and sorted by peak volume (see Table 2[a]). In addition, correlations from the 150 μs CHHC spectrum that were not visible in the 100 μs CHHC spectrum were considered (see Table 2[b]).

Structure calculations. Structure calculations were performed within CNS (Crystallography and NMR System) version 1.1.[33] ^1H-^1H distance constraints obtained from CHHC 2D spectra were represented by square-well potentials. An extended conformer of epothilone B was created as an initial structure and was subsequently subjected to a simulated annealing protocol consisting of three stages: 1) High temperature annealing in cartesian space, in 5000 steps of 0.003 ps at 2000 K. 2) Slow-cool annealing stage in cartesian space, in 5000 steps of 0.005 ps, and temperature reduction from 2000 K to zero in steps of 25 K. 3) Final energy minimization of 2000 steps. During the three stages, force constants k_{HH} of 50, 50, and 25 kcal mol^{-1} Å^{-2} were used. Likewise, the scale factors for the van-der-Waals energy term were set to 0.1, 0.1→4, and 1. An ensemble of 200 structures was generated, starting from different initial velocities. The resulting structures were sorted by total energy, and the 10 lowest energy structures were selected and aligned along the heavy atoms using MOLMOL 2 K.2.[35]

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