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RELATIVE STEREOCHEMISTRY AND SOLUTION CONFORMATION OF THE NOVEL PACLITAXEL-LIKE ANTIMITOTIC AGENT EPOTHILONE A

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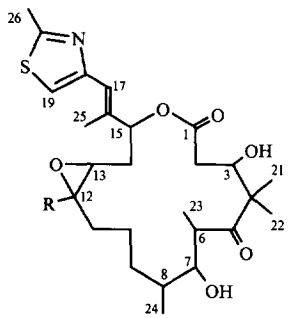
Abstract: A nearly complete set of ^{1}H-^{1}H coupling constants and NOEs were determined for the novel paclitaxel-like antimitotic agent epothilone A from phase sensitive DFQ COSY and NOESY experiments in 75% DMSO-d_6/ D_2O. This data was employed as distance constraints for molecular modeling studies. Molecular dynamic simulations were carried out on a complete series of diastereoisomers, and the results show only two relative stereochemistries to be in agreement with the NOE data.

Introduction

Among the many known antimitotic agents, the epothilones A and B[1] (Figure 1) and discodermolide[2] are the only non-taxane compounds demonstrated to exert their effect by the same microtubule-stabilizing mechanism as the taxanes. The epothilones, which are 16-membered macrolides, isolated from the cellulose-degrading myxobacterium *Sorangium cellulosum*, bear little obvious structural relationship to taxanes. Therefore these compounds represent a key new lead in the search for antimitotic therapeutic agents, and in elucidating the molecular-level mechanism of microtubule stabilization. Although the structures of the epothilones were identified in the original German patent to Hoefle *et al.*,[3] along with their antifungal activity, the stereochemistry of the seven chiral centers was not determined. In this communication, we report NMR and molecular modeling results which allow us to narrow down to two possible relative stereochemistries from among the 64 diastereoisomers.



1 Epothilone A R = H
2 Epothilone B R = CH_3

1a (3*R**, 6*S**, 7*R**, 8*R**, 12*R**, 13*S**, 15*S**)
1b (3*S**, 6*R**, 7*S**, 8*S**, 12*R**, 13*S**, 15*R**)

Figure 1. Structures of the Epothilones.

Table 1. Chemical Shifts, Coupling Constants, and NOE's for Epothilone A[§]

Number	^{1}H Shift[†]	^{1}H Shift[‡]	^{13}C Shift	J_{H-H} [Hz]	NOE
2a	2.41	2.52 dd	38.7	15.4 (2b), 11.0 (3)	8 m/w, 9b m/w, 22 m
2b	2.54	2.43 dd		15.4 (2a), 3.0 (3)	8 m, 9a/10a w, 9b w, 21 w, 22 m/s, 23m
3	4.20	4.08 dd	73.0	11.0 (2a), 3.0 (2b)	2a w, 2b m/w, 6 m, 21 m, 22 m, 23 w
6	3.23	3.15 dd	45.5	7.0 (23), 9.0 (7)	2a m, 2b m, 8 m, 9a/10a m, 22 s, 23 s
7	3.79	3.56 m	76.4	9.0 (6), vs (8)	6 m, 8 s, 9a/10a w, 23 m/s, 24 s
8	1.76	1.37 m	35.8	l (9a), s (9b), vs (7)	
9a	1.4	1.21 m	29.8	l (9b), l (8), s (10a), s (10b)	21 m
9b	1.52	1.44 m		l (9a), s (8), s (10a), l (10b)	8 m, 9a s, 10a s, 22 m, 23 m/s
10a	1.4	1.16 m	24.0	l (10b), s (11a), l (11b)	24 s
10b	1.4	1.57 m		l (10a), l (11a), s (11b)	8 w, 9b/11a m/w, 10a s, 24 m
11a	1.42	1.41 m	27.4	s (10a), l (10b), l (11b), l (12)	[overlaps with 9b]
11b	1.7	1.73 m		l (10a), s (10b), l (11a), s (12)	10a m, 10b w, 11a s
12	2.91	2.94 ddd	57.6	l (11a), s (11b), ~4 (13)	10a m, 10b m, 11a m, 11b m
13	3.03	3.15 ddd	55.1	~4 (12), l (14a), s (14b)	12 s, 14a m/w, 14b m
14a	1.88	1.83 dd	32.4	l (13), 15.0 (14b), 9.9 (15)	11a m, 25 m
14b	2.14	2.16 dd		s (13), 15.0 (14a), vs (15)	11a m, 14a s, 25 m
15	5.43	5.32 br d	76.6	9.9 (14a), vs (14b)	12 w, 13 s, 14a w, 14b s, 25 m
17	6.60	6.56 br s	119.4	-	15 s, 25 m
19	6.98	7.38 s	117.8	-	17 m, 25 s
21	1.10	0.99 s	20.9	-	
22	1.37	1.29 s	23.0	-	
23	1.18	1.12 d	17.0	7.0 (6)	
24	1.01	0.96 d	18.7	7.0 (8)	
25	2.09	2.09 br s	14.9	-	26 w
26	2.70	2.69 s	18.8	-	
3-OH	3.97				
7-OH	2.58				

[§]Carbon shifts of protonated carbons extracted from HMQC data. Where J's could not be measured, they were classified from the footprint of phase sensitive DQF-COSY crosspeaks as large (l), 8-10 Hz; small (s), 3-4 Hz; very small (vs), <2.5 Hz. NOE's were classified as strong (s), medium/strong (m/s), medium (m), medium/weak (m/w), and weak (w).

[†]In $CDCl_3$, as reported in ref. 3 and confirmed in our laboratory.

[‡]In 75% DMSO-d_6/25% D_2O.

Nuclear Magnetic Resonance Studies

An authentic 1 mg sample of epothilone A was a gift of Dr. Michael Goetz at Merck Research Laboratories (Rahway, NJ). Preliminary ^{1}H NMR examination of the compound in $CDCl_3$ confirmed the assignments and shifts given in the patent;[3] however, extensive overlap, especially of the protons on C9-C11, precluded complete assignment in that solvent. Based on our experience with conformational analysis of taxanes,[4] we collected phase sensitive DQF COSY and NOESY data in 75% DMSO-d_6/25% D_2O (Bruker AM-500). In this solvent mixture, the spectral dispersion was significantly improved and all of the protons could be assigned (Table 1). Many of the coupling constants could be measured directly, but overlap was still substantial between 1.1 and 1.5 δ. However, the improved dispersion permitted reliable estimation of those couplings which could not be measured directly from the footprint of DQF COSY crosspeaks, for a total of 26 constraints. As for the taxanes, NOESY data acquired at sub-ambient temperatures (-20° C) gave all positive crosspeaks for this "medium sized" molecule (molecular weight 493). 61 NOEs were identified; a few involving protons within the C9-C11 region were obscured by overlap, but the presence of unbroken *J*-coupling in this region allow its conformation to be reasonably well described nonetheless.

Unlike the case of the taxanes, there were no dramatic conformational differences indicated between polar and nonpolar solvents. However, the increased chemical shift dispersion, and changes to some coupling constants in DMSO-d_6/ D_2O, seemed to indicate there was less conformational averaging occurring in this solvent. Besides the 9-11 region, the C1 ester bond was affected: the 2a, b-3 and 14a, b-15 *J*-couplings become more unlike in DMSO-d_6/ D_2O. An interesting comparison is to another family of antimitotic agents, the cryptophycins; these cyclic depsipeptides were recently described to have very similar solvent-dependent changes in *J*-couplings,[5] which were related to changes in preferred conformation and conformational averaging at ester bonds. Consequently, we employed the DMSO-d_6/ D_2O data as constraints in the molecular modeling, as we had a nearly complete set of couplings and NOEs in this solvent, and the data seemed to indicate that the possibility of characterizing a virtual conformation was reduced compared to using the $CDCl_3$ data. Also, there is support in the literature for the idea that the conformation(s) present in polar solvent are more relevant to the issue of bioactivity.[6]

Molecular Modeling Studies

Molecular modeling techniques were applied to determine possible relative stereochemistries of epothilone A by performing molecular dynamic simulations on a series of stereoisomers using distance constraints obtained from the NOE data. Our approach began by determining the most probable geometry of the epoxide ring. The approximate 4 Hz $J_{12,13}$ value supported the presence of a *cis*-substituted epoxide, which meant that the absolute stereochemistry at C12 and C13 had to either be 12*R*, 13*S* or 12*S*, 13*R*. The 12*R*, 13*S* stereochemistry was arbitrarily chosen as the starting point to construct models of all 32 (2^5) possible stereoisomers based upon the 5 remaining stereocenters (it was expected that using the other combination of stereochemistries for the epoxide would result

in obtaining the same overall relative stereochemistry of epothilone A). Using the molecular modeling software package SYBYL,[7] models of the 32 stereoisomers were constructed using the Karplus relationship[8] to derive the torsion angles from the ^{1}H-^{1}H coupling constants.

NOEs were classified as strong, medium/strong, medium, medium/weak, and weak. Each NOE category was then assigned a distance range: 1.5-2.8 Å, 2.8-3.5 Å, and 3.5-5.0 Å corresponding to strong , medium, and weak NOEs, respectively. Six key distance constraints derived from the NOEs [H-3/H-6 (m), H-6/H-7 (m), H-6/H-8 (m), H-7/H-8 (s), H-12/H-13 (s), and H-15/H-17 (s)] were applied to each of the models, and the structures were subjected to an energy minimization using the standard Tripos force field[9] without electrostatics. Partial atomic charges were then calculated using the Gasteiger-Hückel method, and each stereoisomer was submitted for a 100 ps molecular dynamics simulation while collecting data every 50 fs. Each simulation was calculated in vacuo at 400 K with a time step of 1 fs and a temperature coupling factor of 100 fs. One hundred low-energy conformers were collected for each of the 32 dynamic simulations, and each set of conformers was subjected to an energy minimization using the supplemental SPL Sysmin macro of SYBYL.[10] A root-mean-square (RMS) deviation of 0.175 was used with this routine which allowed only those minimized conformers with a larger RMS deviation from the previous minimized structures to be accepted and saved as a unique conformation. This procedure provided a total of 376 unique conformations for the 32 stereoisomers. All unique structures were then subjected to an additional energy minimization without enforcing the NOE distance constraints.

A SYBYL molecular spreadsheet containing the unique conformations obtained from the dynamic simulations was used to calculate all torsion angles and interproton distances of interest. Each conformation was carefully examined for its consistency with the NOE data. This analysis led to the finding that only two of the possible 32 stereoisomers were consistent with the NOE data, that is those isomers having the 3*R*, 6*S*, 7*R*, 8*R*, 12*R*, 13*S*, 15*S* (**1a**) and 3*S*, 6*R*, 7*S*, 8*S*, 12*R*, 13*S*, 15*R* (**1b**) configurations. Their calculated vicinal proton coupling constants and key interproton distances are shown in Tables 2 and 3. Surprisingly, except for the epoxide centers, these two isomers have opposite configurations at each of the chiral centers.

While the 4 Hz $J_{12,13}$ value tended to support the presence of a *cis* epoxide, we were still concerned with its intermediate value. We believed the possibility of a *trans* epoxide could not be completely ruled out, and therefore repeated similar calculations with the 12*R*, 13*R* *trans* epoxide. This procedure provided the same results as to the relative stereochemistry of the five chiral centers besides the epoxide center; and furthermore, it showed the *trans* epoxide to be inconsistent with the NOE data in the C11-C14 region.

In summary, the results of this molecular dynamics/NOE distance-constraint method suggest the relative stereochemistry of epothilone A to be either that of diastereoisomer **1a** or **1b** (Figure 2).

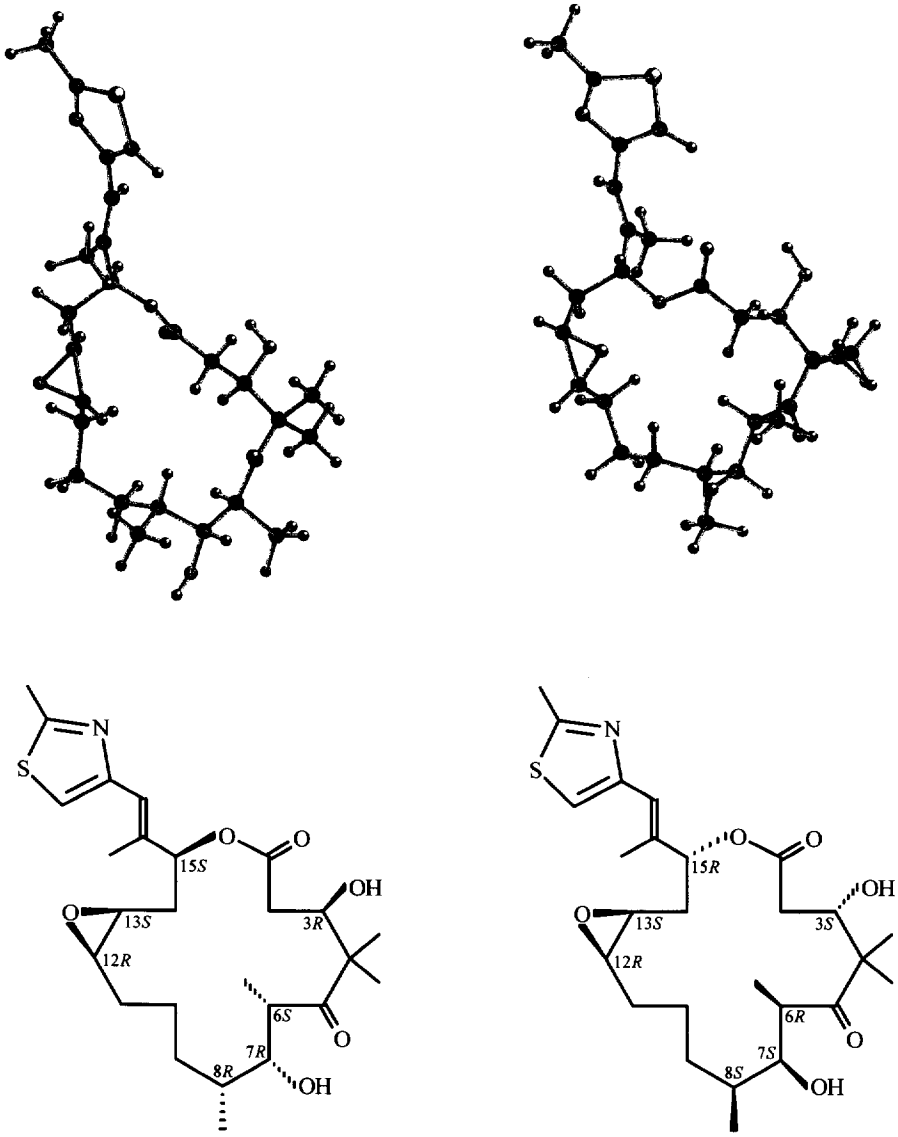


Figure 2. Structures of diastereoisomers **1a** (left) and **1b** (right).

Table 2. Proton Coupling Constants (*J*, Hz)

J_{H-H}	obs.	calcd. (**1a**)	calcd. (**1b**)
2a-3	11.0	10.07	9.01
2b-3	3.0	2.00	1.37
6-7	9.0	10.26	10.24
7-8	<2.5	1.99	1.38
8-9a	8-10	10.19	10.11
8-9b	3-4	2.03	1.72
11a-12	8-10	9.04	9.39
11b-12	3-4	1.37	1.45
13-14a	8-10	10.26	7.09
13-14b	3-4	2.53	6.70
14a-15	9.9	10.22	10.02
14b-15	<2.5	2.25	1.79

Table 3. Interproton Distances (Å)

NOE	Distance (**1a**)	Distance (**1b**)
H-2b/H-8 (m)	2.76	2.81
H-2b/H-9b (w)	3.55	3.49
H-2b/H-6 (m)	3.52	2.79
H-3/H-6 (m)	2.77	2.76
H-6/H-9a (m)	3.40	3.52
H-10a/CH_3-24 (s)	2.30	2.31
H-11a/H-14a (m)	2.30	2.55
H-12/H-10b (m)	2.79	2.67
H-12/H-13 (s)	2.55	2.57
H-13/H-15 (s)	2.65	3.00
H-15/H-17 (s)	2.33	2.43
H-17/H-19 (m)	3.52	3.53

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