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TAXOL: QUANTITATIVE INTERNUCLEAR PROTON-PROTON DISTANCES IN $CDCl_3$ SOLUTION FROM nOe DATA: 2D NMR ROESY BUILDUP RATES AT 500 MHz

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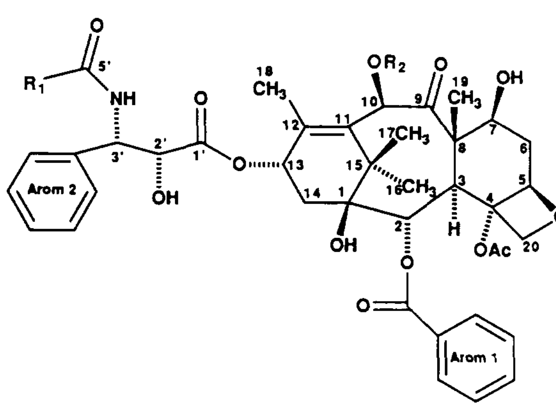
ABSTRACT.—Quantitative nmr internuclear proton-proton distance measurements obtained by observation of the initial buildup rates of nOe's in 2D ROESY spectra of taxol [**1**] in $CDCl_3$ are reported. A comparison to the X-ray crystal structure of taxotere [**2**] is made, and the results are discussed in terms of previous studies of structure-activity relationships.

Taxol [**1**] is a promising antineoplastic agent currently in phase II clinical trials for ovarian and breast cancer. Its mechanism of action involves interference with tubulin depolymerization (1). Recently we reported ^{1}H- and ^{13}C-nmr assignments for taxol and two related compounds (2). Using these assignments, continued studies of taxol in $CDCl_3$ at 500 MHz resulted in quantitative internuclear proton-proton distances calculated from the initial buildup rates of nOe's measured by ROESY.

The quantitative estimation of internuclear distances in molecules of small to intermediate size is properly approached by ROESY spectroscopy because many nOe crosspeaks (where $\omega\tau_c \simeq 1$) would be expected to be diminished or lost in a NOESY spectrum. The choice of a 2D nOe experiment over 1D kinetic nOe experiments is justified because spectral crowding occurs in the 1D ^{1}H-nmr spectrum of taxol even at 500 MHz. The extraction of distance information from ROESY spectra depends on being able to obtain an accurate estimate of the initial buildup rate of the nOe intensity of a crosspeak. That is, crosspeak volumes must be measured at times short compared to competing relaxation events, so that the initial rate approximation holds (3). There are two advantages for using ROESY to determine distance information for molecules the size of taxol compared to similar approaches being used to obtain macromolecular structural information (4–6): (1) spin diffusion is less significant for molecules of shorter τ_c, and (2) the relaxation times



1 R_1=Arom 3, R_2=Ac
(Phenyl)
2 R_1=O*t*Bu, R_2=H

of smaller molecules are considerably longer. These advantages allow measurement of the nOe buildup rates for a longer period of time before the initial rate approximation fails. ROESY spectra, however, do present various difficulties in interpretation (7,8). The measurement of nOe intensities vs. time instead of single point nOe's proved to be crucial in overcoming many of these problems. Many cases arose in which an individual ROESY spectrum appeared to give an nOe correlation (an observed crosspeak), but the time dependence of the crosspeak volume showed that distance information should not be extracted from that crosspeak because, for example, negative or non-linear buildup curves resulted.

A particularly difficult complication is represented by false transverse nOe crosspeaks, in which an nOe is transferred via scalar coupling correlation through a third peak. In taxol, for example, H_a-20 and H_b-20 are geminally coupled, and H_b-20 is much closer to Me-19 than H_a-20. As expected, the "distance" measured for H_a-20/Me-19, 2.7 Å, is much too small and thus represents a transferred nOe. Another example of this phenomenon is seen in an apparent distance of 3.22 Å for H-3′/*m*-Ph2; the nOe is clearly transferred through *o*-Ph2. The approach used with the present data was to flag correlations of this type in Table 1 only in cases where other data such as scalar couplings or primary structural considerations made the interpretation quite obvious. For example, for H_a-20/H_b-20, the "W" coupling of 0.8 Hz between H_a-20 and H-3 provides additional evidence that H_a-20 is farther from Me-19 than H_b-20.

Another common 3-spin interaction which complicates 1D nOe spectroscopy, relayed nOe (spin A to spin C through spin B), appears in ROESY spectra in phase opposite to that of real nOe crosspeaks and can be easily ruled out in cases where the relayed nOe is the dominant process producing the correlation.

Estimated initial buildup rates, the proton-proton distance parameters estibated from them, and the corresponding proton-proton distances from the taxotere [**2**] X-ray crystal structure (9) are shown in Table 1A (for proton-proton connectivities in the baccatin core region of taxol) and Table 1B (all other connectivities). The baccatin core region of taxol [**1**] is expected to be essentially rigid and to be identical in structure to that of taxotere [**2**]; the motion of all of the protons in the core region is expected to be characterized by a single correlation time. The remaining parts of taxol are expected to involve flexibility. In the presence of structural flexibility, nOe measurements can rigorously be used only to indicate the closest approach of a proton-proton pair, since in theory the nOe represents a time average, weighted by the inverse sixth power of the interproton distance, over all conformations of the molecule.

Of the 30 baccatin core distances shown in Table 1A, 24 agree remarkably well with the taxotere structure. A numerical measure of the degree of fit cannot be given because of the ambiguity in the distance that should be assigned to methyl protons; nevertheless it is clear that nearly all of the distances are within about 0.3 Å of the crystal structure. The remaining six distances are all noticeably shorter than in the crystal data; each was interpreted to be a false transverse nOe as discussed earlier. It is concluded that (a) the nOe data confirm in detail that the structure of the baccatin core of taxol is essentially identical to that of taxotere from the crystal structure, and (b) the distances extracted, with the caveat that careful objective interpretation of the data is required to isolate cases of false transfer nOe, are quite accurately determined by the buildup method.

In addition to connectivities involving only the rigid baccatin core, some 17 other proton-proton connectivities

TABLE 1. Distance Data for Taxol Compared to Taxotere X-ray Crystal Data.[a]

	Buildup rate	Distance calcd	Taxotere Crystal Structure	
			nearest H	center C
A. Proton-proton connectivities in the baccatin core region				
H-2[b] H_a-20	0.77	3.21	4.00	
H-2 H_b-20	1.00	3.07	2.83	
H-2 Me-16	9.09	2.28	1.94	2.52
H-2 Me-19	6.68	2.40	2.27	2.77
H-3 H-5	0.89	3.13	3.60	
H-3 H-7	11.61	2.04	2.19	
H-3 H-10	3.16	2.53	2.89	
H-3 Me-16	0.55	3.63	3.45	3.82
H-3 Me-18	2.45	2.79	2.87	3.22
H-3 Me-19	0.93	3.34	3.69	3.38
H-5 H_a-6	7.77	2.18	1.95	
H-5 H_b-6	1.54	2.86	2.57	
H-5[b] Me-19	1.13	3.22	3.64	4.19
H_a-6 H-7	6.56	2.25	2.84	
H_a-6 H_b-6	30.42	1.74	1.74	
H_b-6 Me-19	6.79	2.39	2.40	2.76
H-7 Me-18	2.12	2.90	2.56	3.42
H-7 Me-19	0.85	3.38	3.63	3.38
H-7 H-10	11.82	2.04	2.54	
H-10 H-3	3.16	2.54	2.89	
H-10 Me-18	12.00	2.17	2.19	2.48
H-13 Me-17	11.83	2.18	1.64	2.48
H-13 Me-18	1.19	3.19	3.11	3.12
H_a-14[c] H-3	5.27	2.39	2.22	
H_b-14[b,c] H-3	1.71	2.81	3.59	
Me-16 Me-17	10.72	2.48	2.38	2.44
Me-16 Me-19	2.00	3.29	2.65	4.19
H_a-20[b] H_a-6	0.82	3.18	4.79	
H_a-20[b] Me-19	3.03	2.56	3.36	4.12
H_b-20[b] H_a-6	0.70	3.26	4.68	
H_b-20 Me-19	5.15	2.51	2.06	2.73
B. All other connectivities				
4-Ac H-2′	2.96	2.75	3.98	4.30
4-Ac H-3′	3.45	2.67	2.64	3.58
4-Ac *o*-Ph1	0.84	3.42	3.13	4.06
4-Ac *o*-Ph2	0.96	3.35	2.70	3.53
H-2′ H-3′	5.61	2.31	2.33	
H-2′ Me-18	0.98	3.35	2.54	3.53
H-2′ *o*-Ph2	1.37	2.92	2.75	2.89
H-3′[b] *m*-Ph2	0.76	3.22	4.60	
H-3′ *o*-Ph2	2.40	2.66	2.42	
3′-NH H-2′	0.76	3.23	3.68	
3′-NH H-3′	1.77	2.79	2.90	
3′-NH *o*-Ph2	1.72	2.91	2.08	
3′-NH *o*-Ph3	3.10	2.54	na	
10-Ac H-13	1.43	3.10	na	
10-Ac Me-16	1.09	3.64	na	
H_a-20 *o*-Ph1	1.04	3.06	3.37	
H_b-20 *o*-Ph1	0.69	3.27	4.04	

[a]Observed initial buildup rates are in arbitrary units/sec. Calculated distances and taxotere reference distances are in Å.

[b]Dominated by false transverse nOe (see text).

[c]H_a-14 and H_b-14 correspond to H_b-14 and H_a-14 in the taxotere X-ray structure (9) because our designations were made on the basis of chemical shift. Coincidentally all other ab pairs were the same.

were observed (Table 1B). As noted previously, considerable flexibility may be expected to be reflected in these nOe data. Nevertheless, out of the 14-proton-proton nOe buildups measured that can be compared to the taxotere crystal structure, 10 yield estimations of distance that are within 0.45 Å to the taxotere structure. One of the remaining connections, H-3′ to *m*-Ph2, is another example of an nOe transferred (via *o*-Ph2) to another proton via a scalar coupling pathway. Probably the H_b-20/*o*-Ph1 connection is also of this type, but there is no objective basis on which to make this assignment. This leaves only 3′-NH/*o*-Ph2 and 4-Ac/H-2′ nOe's as giving distances substantially different from the taxotere crystal structure. The data, taken as a whole, suggest that the taxol molecule in $CDCl_3$ solution adopts a conformation very similar to the taxotere crystal structure, that is to say, that a substantial fraction of the conformations contributing to the time average taxol structure in $CDCl_3$ solution are close to the coordinates defined by the taxotere X-ray crystal structure. Furthermore, this may represent essentially a single structure. To test this latter hypothesis, molecular modelling calculations are currently being carried out.

The connections shown in Table 1B are consistent with a structure in which the sidechain (C-2′, C-3′) and the phenyl rings fold together to place C-2′/C-3′ close to the oxetane ring system. This is interesting in light of structure activity studies of taxol (10). In particular, the nature of the polar groups at C-2′ and C-3′ is not important to activity, but the stereochemistry at C-2′ is important, and a very large change in activity is observed when the C-2′ and C-3′ groups are interchanged (10). In addition, the opening of the oxetane ring drastically reduces cytotoxicity, while loss of the 3′ *N*-benzoyl group results in loss of activity (10). This suggests that the crucial function of the sidechain backbone is to provide conformational flexibility sufficient to allow the achievement of a folded structure in which the sidechain and the phenyl groups closely approach the oxetane ring, whether or not the structure thus attained has a long lifetime.

In summary, a very straightforward process of obtaining initial buildup rates of nOe crosspeaks for taxol has provided a set of distance information suitable for testing various hypotheses concerning the dynamic nature of taxol. Interesting correlations exist between the nOe results and structure-activity relationships. It should be emphasized that the distance parameters were obtained in this study with a minimum of mathematical manipulations; for example, no iterative fit of the nOe data to a multispin nOe calculation was used, as is currently done for protein nOe structural studies (11). This approach was successful largely as a consequence of the fact that spin diffusion, or nOe crosstalk, is much less important in small molecules than in macromolecules with large τ_c, and that the observation of the buildup process allows the immediate rejection of various kinds of spurious nOe connections.

EXPERIMENTAL

^{1}H-nmr spectroscopy (500 MHz) was performed on a Varian VXR500S spectrometer equipped with a SUN 4/110 workstation. ^{1}H chemical shifts are reported relative to TMS = 0.00 ppm. All ROESY spectra were obtained nonspinning with temperature maintained at 27° with a Varian variable temperature controller using a sample concentration of 26 mg/ml. ROESY spectra were obtained with States-Haberkorn phase cycling. A Kessler spin lock of 30° pulses was employed (12). The 90° pulse width for all proton pulses was 8.5 μsec. A post acquisition delay of 2.0 sec proved adequate; increasing this delay to 5.0 sec did not alter the data appreciably. A homospoil pulse was used at the beginning of the pulse sequence to suppress effects of incomplete longitudinal recovery. For each 2D spectrum, 512 increments of 1024 complex points were obtained and zero filled to 2048 × 2048 complex points.

Mixing times were arrayed over 50–300 msec in a single experiment (at least five data points).

Eight steady-state scans were performed before each new mixing time experiment. Individual t_1 increments were interleaved to obtain the 2D data set for a single mixing time. The raw data for each mixing time were obtained separately; however, the order of the mixing times used was scrambled to reduce time-dependent effects, i.e., 50 msec, 300 msec, 100 msec, 200 msec, 150 msec. The buildup curves were significantly nonlinear after 200 msec; thus initial buildup rates were estimated by a linear fit of the crosspeak volumes vs. mixing times where the mixing time was less than 160 msec (at least 3 data points). Distance data were calculated by assigning a proton-proton distance of 1.74 Å to the buildup rate obtained for the geminal pair 6a/6b (from taxotere X-ray crystal structure) and the assumption that buildup rates are inversely proportional to the sixth power of the inter-proton distance. Crosspeak volumes were scaled to compensate for the theoretical volume expected for equivalent protons (13). Distances shown for the taxotere crystal structure involving methyl groups are shown to the methyl carbon involved and to the nearest proton of the methyl group.

The errors associated with the calculated distances in Table 1 are difficult to assess. Attempts to analyze the accuracy of a buildup [for example, Van de Ven *et al.* (14)] suggest that the error is roughly ± 0.3 Å for protons separated by less than 3.0 Å, while the error increases sharply for longer distances. This, however, assumes that one is able to make a confident assignment of crosspeaks to Overhauser connectivities and that the correlation time of the internuclear vector of a particular proton pair is similar to the correlation time of the internuclear vector of the proton pair providing the distance reference.

A referee brought to our attention that a report has recently appeared which seems to be in general agreement with our results (15). In this report laboratory frame (NOESY) experiments in CH_2Cl_2 were performed; these did not involve obtaining buildup curves.

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