Molecular Modeling, NMR Spectroscopy, and Conformational Analysis of 3',4'-Anhydrovinblastine

C. Webb Andrews[◊], James Wisowaty[#], Ann O. Davis, Ronald C. Crouch and Gary E. Martin*

Division of Organic Chemistry[◊], Chemical Development Laboratories[#], and Bioanalytical Sciences, Wellcome Research Laboratories*
Burroughs Wellcome Co., Research Triangle Park, NC 27709

Received June 22, 1994
Revised January 17, 1995

The assignment of the proton spectrum of 3',4'-anhydrovinblastine is reported. Assignments are made for several protons for which only approximate assignments were available previously. Homonuclear TOCSY and ROESY spectra were utilized in conjunction with HMQC and HMBC spectra in making the assignment. Correlations in the ROESY spectrum suggested a preferred conformation of the cleavamine (upper) portion of 3',4'-anhydrovinblastine in which the 21-methyl of the 20/21 ethyl group of the vindoline (lower) portion is in proximity to the H14' and 16'-NH resonances of the cleavamine. In a Monte Carlo search, the global minimal energy structure was oriented with the 16-methoxyl group oriented toward the H14' and 16'-NH resonances. Two other structures, the second and tenth lowest in energy, 0.2 kJ and 8 kJ higher in energy, respectively, brought the 21-methyl group in proximity to the H14' and 16'-NH resonances in a fashion consistent with the ROESY data. The preferred solution conformation of 3',4'-anhydrovinblastine is consistent with the reported solution conformation of vinblastine.

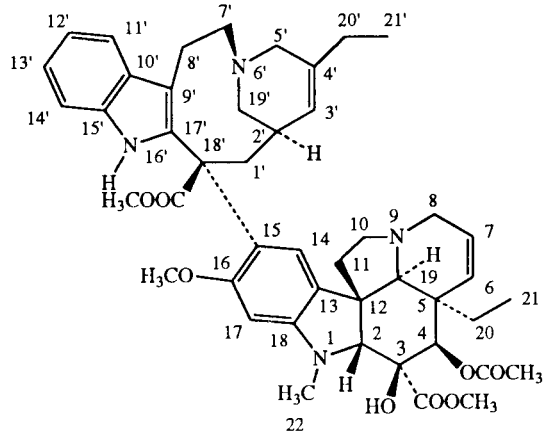
J. Heterocyclic Chem., **32**, 1011 (1995).

Introduction.

During the course of developing the bisindole alkaloid anticancer drug Navelbine® (vinorelbine), we had occasion to complete total proton and carbon nmr spectral assignments of the parent compound [1], a number of degradants [2], and synthetic route markers. One of the compounds studied was 3',4'-anhydrovinblastine (**1**, AHVLB, IUPAC numbering). Previous work by Szantay and co-workers [3] reported nearly complete proton resonance assignments and a total assignment of the ^{13}C nmr spectrum of **1**, as well as some analysis of the preferred conformation of the cleavamine subunit of the molecule. A carbon spectral assignment of **1** was reported by Kutney *et al.* [4], who have also dealt with the flavine coenzyme mediated photo oxidation of **1** and its conversion to an unstable dihydropyridinium intermediate which may play a crucial role in bisindole alkaloid biosynthesis. In light of recent interest in **1**, we wish to report the results of our spectral and molecular modeling studies of **1** which have allowed us to complete the proton assignments of Szantay *et al.* [3] and to demonstrate that the preferred solution conformation of **1** in chloroform is very similar to that of vinblastine in both the solution [5,6] and crystalline states [7,8].

Results and Discussion.

Assignment of the proton and carbon resonances of the vindoline (lower) subunit of 3',4'-anhydrovinblastine (**1**) followed in a straight-forward manner from the interpretation of COSY, HMQC, and HMBC spectra. Data were acquired at -10° to aid in stabilizing the sample. Proton and carbon resonance assignments are tabulated and compared for the vindoline subunit in Tables 1 and 2 with those reported by Szantay *et al.* [3]. In general the proton resonances were sharp and well resolved (see Figure 1), making it quite easy to follow connectivity networks in the COSY spectrum shown in Figure 2A.

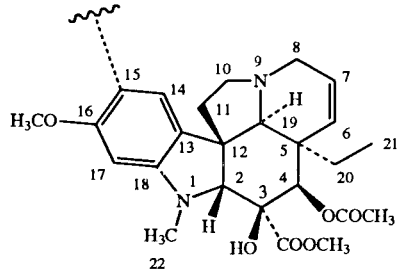


1

In contrast, proton resonances at the 5'-, 7'-, 8'-, and 19'-positions in the cleavamine (upper) subunit were significantly broadened (see for example the region of the proton reference spectrum from 3.2-3.8 ppm), suggesting internal motion in the nine-membered azanonine ring. As a direct consequence of broadening in the proton spectrum, the COSY spectrum was of minimal value in establishing proton-proton connectivities in the cleavamine subunit. To circumvent this problem, homonuclear TOCSY spectra were

Table 1

Proton Resonance Assignments for the Vindoline "Half" of 3',4'-Anhydrovinblastine (1) in Deuteriochloroform



Position		Chemical Shift (δ ppm)	
Biogenetic	IUPAC	Szantay *et al.* [3]	Present Report
2	2	3.72	3.73
16-OH	3-OH	9.88	10.05
17	4	5.47	5.44
15	6	5.86	5.30 [a]
14	7	5.31	5.85 [a]
3	8	3.37	3.36
		2.83	2.86
5	10	3.23	3.28
		2.45	2.51
6	11	2.15	2.13
		1.80	1.77
9	14	6.62	6.52
12	17	5.47	5.44
21	19	2.68	2.66
19	20	1.79	1.77
		1.35	1.33
18	21	0.81	0.78
1-NMe	1-NMe	2.71	2.71
16-$COOCH_3$	3-$COOCH_3$	3.80	3.79
17-$OCOCH_3$	4-$OCOCH_3$	2.12	2.10
11-OCH_3	16-OCH_3	3.82	3.82

[a] These assignments were reversed from those reported by Szantay *et al.* [3] on the basis of correlations from the H7 resonance to the H8 methylene protons observed in the ROESY spectra.

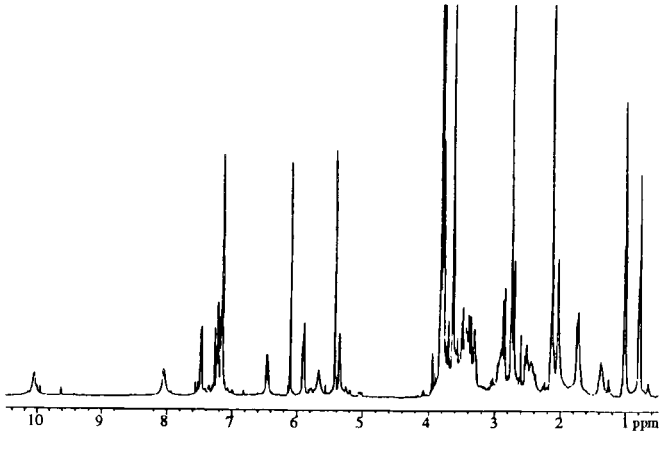
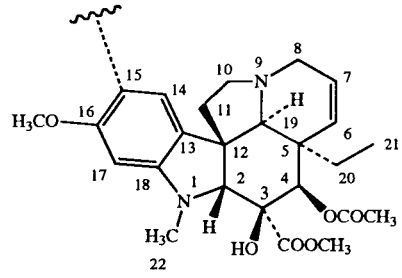


Figure 1. Proton reference spectrum of 3',4'-anhydrovinblastine (1) recorded at 500 MHz in deuterochloroform at -10° C.

Table 2

Carbon Resonance Assignments for the Vindoline "Half" of 3',4'-Anhydrovinblastine (1) in Deuteriochloroform



Position		Chemical Shift (δ ppm)	
Biogenetic	IUPAC	Szantay *et al.* [3]	Present Report
2	2	83.3	82.2
16	3	79.7	79.2
17	4	76.4	75.7
20	5	42.6	52.0
15	6	130.0	130.1
14	7	124.5	124.5
3	8	50.2	49.6
5	10	50.2	49.1
6	11	44.6	44.3
7	12	53.3	52.8
8	13	122.8	123.0
9	14	123.6	122.4
10	15	121.2	121.6
11	16	158.0	157.2
12	17	94.2	93.4
13	18	152.7	152.6
21	19	30.8	30.2
1-NMe	1-NMe	38.4	37.5
16-$COOCH_3$	3-$COOCH_3$	170.9	170.7
16-$COOCH_3$	3-$COOCH_3$	52.2	52.0
17-$OCOCH_3$	4-$OCOCH_3$	171.7	170.7
17-$OCOCH_3$	4-$OCOCH_3$	21.2 [a]	20.9
11-OCH_3	16-OCH_3	55.8	55.4

[a] No chemical shift for this carbon was reported in the paper of Szantay *et al.* [3]; the chemical shift listed is from the work of Kutney *et al.* [4] and is shown for comparison only, as no solvent was specified in that study.

acquired with mixing times of 12 and 24 msec. The TOCSY spectrum acquired with a mixing time of 12 msec is shown in Figure 2B. These data were, compared to those of the COSY spectrum, useful in identifying the 5'-, 7'-methylene resonant pairs. The 8'-methylene resonances could not be readily identified even from the TOCSY data. The 19'-methylene resonances were ultimately assigned from correlations in the HMQC spectrum (not shown), by comparing carbon chemical shifts to those reported by Szantay *et al.* [3]. Ultimately, one of the H8'-methylene resonances was located at 3.41 ppm *via* a rOe correlation from the H11' aromatic proton in the ROESY spectrum shown in Figure 3. The remaining H8' proton was located at 3.05 ppm from the very weak H8'/C8' correlations in the HMQC spectrum.

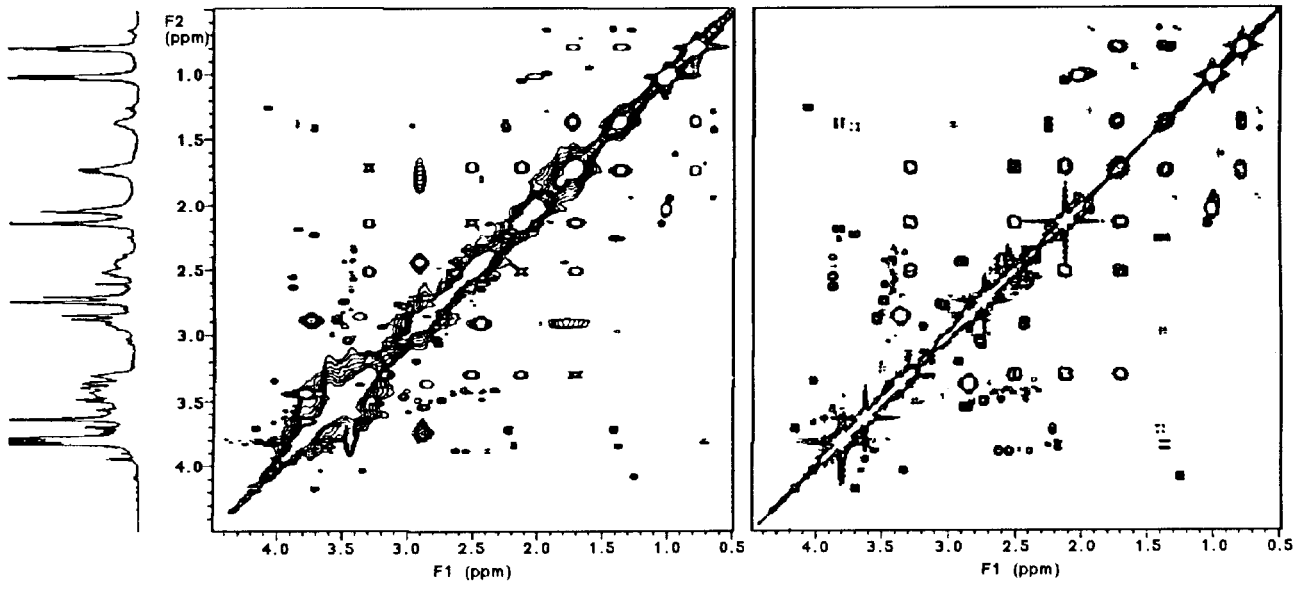


Figure 2. Comparison of the aliphatic region of the COSY and TOCSY spectra of 3',4'-anhydrovinblastine (**1**) in deuteriochloroform at -10° . The COSY spectrum (**A**) shows sharp, well defined resonances for the vindoline portion of the molecule and virtually no geminal or vicinal connectivity information for the cleavamine subunit (in the range of 3.2-3.8 ppm). In contrast, the TOCSY spectrum (**B**) shows a number of connectivities in the region from 3.2-3.8 ppm which are essential to the assignment of the cleavamine proton resonances of the azononine ring.

The rOe cross peak correlating H11', which resonates at 7.47 ppm, and the H8' proton at 3.41 ppm is reasonable on the basis of molecular modeling and the work of Szantay *et al.* [3]. The cleavamine portion of the molecule was initially modeled with an *o*-methoxyphenyl attached to the 18'α position to satisfy the valence requirement of the 18'α position and to roughly approximate the presence of the vindoline subunit. Using this model, H11' was located 2.3Å from H8'a (Figure 4), clearly accounting for the intense rOe cross peak observed in the spectrum shown in Figure 3.

Despite considerable overlap in the region from 3.3 to 3.7 ppm of the proton spectrum of **1**, it was possible to unequivocally locate the 7'-methylene protons at 3.61 and

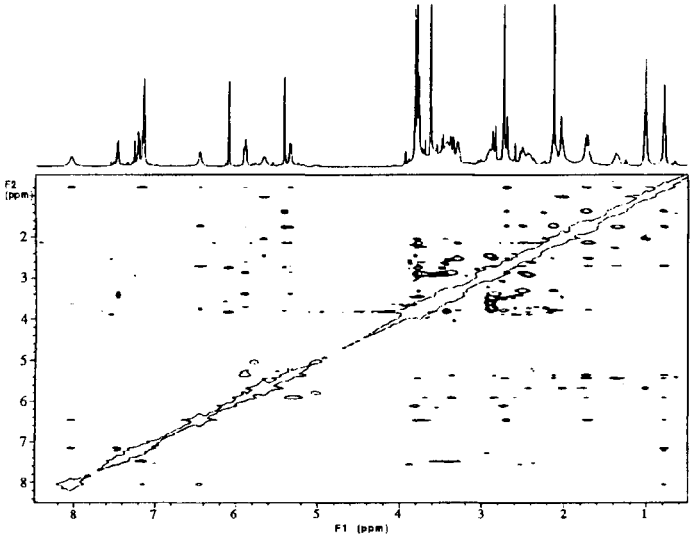


Figure 3. ROESY spectrum of 3',4'-anhydrovinblastine (**1**) recorded in deuteriochloroform at -10° with a mixing time of 250 msec and a B_1 spin-lock field of 3.2 KHz. The diagonal responses and open off-diagonal responses defined by a single contour are negatively phased; off-diagonal responses defined by numerous contours have positive intensity and define rOe correlations.

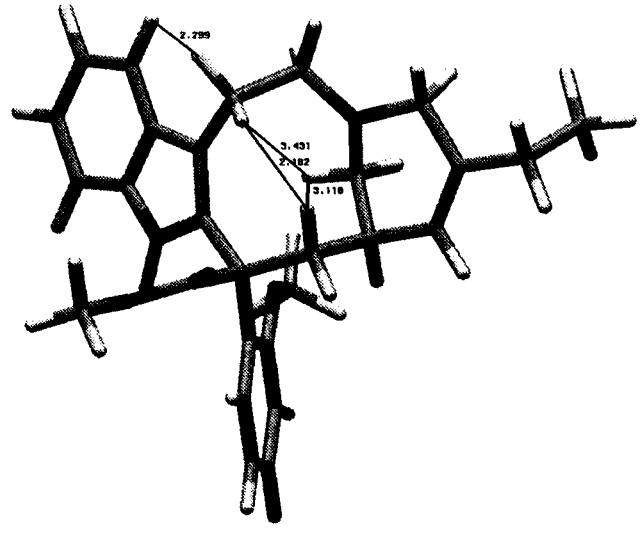
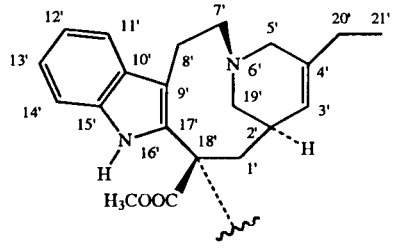


Figure 4. Minimized structure of the cleavamine subunit substituted at the 18'α-position with an **o**-methoxyphenyl substituent to roughly approximate the presence of the vindoline subunit. Distances from the H8' geminal methylene resonances to several resonances in proximity are shown.

Table 3

Proton Resonance Assignments for the Cleavamine "Half" of 3',4'-Anhydrovinblastine (1) in Deuteriochloroform



Position		Chemical Shift (δ ppm)	
Biogenetic	IUPAC	Szantay *et al.* [3]	Present Report
17'	1'	3.04	2.92
		2.40	2.44
14'	2'	1.30	1.78
15'	3'	5.47	5.67
21'	5'	3.52	3.79
		3.28	3.44
5'	7'	~3.4	3.61
		~3.4	3.44
6'	8'	3.41	3.39
		3.05	3.13
9'	11'	7.53	7.47
10'	12'	~7.14	7.16
11'	13'	~7.14	7.22
12'	14'	~7.14	7.15
1'	16'-NH	8.05	8.04
3'	19'	3.31	3.73
		2.58	2.88
19'	20'	1.93	2.04
18'	21'	0.99	1.01
16'-$COOCH_3$	18'-$COOCH_3$	3.62	3.62

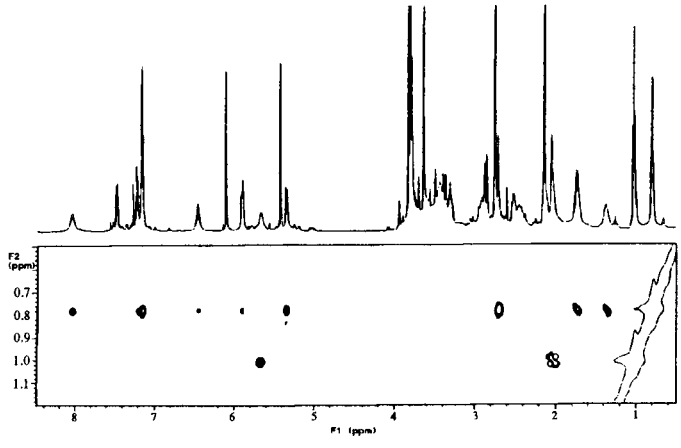


Figure 5. Expanded segment of the ROESY spectrum shown in Figure 3 showing correlations from the 21-methyl group of the vindoline subunit. The correlations of interest are those at 8.04 and 7.15 ppm which correspond to rOe correlations to the 16'-NH and 14'-aromatic resonances, respectively. For the observed correlations to be found in this spectrum, the preferred conformation of 3',4'-anhydrovinblastine in solution must bring the 21-methyl in proximity to the indole resonances of the cleavamine subunit (see also Figures 7 and 8).

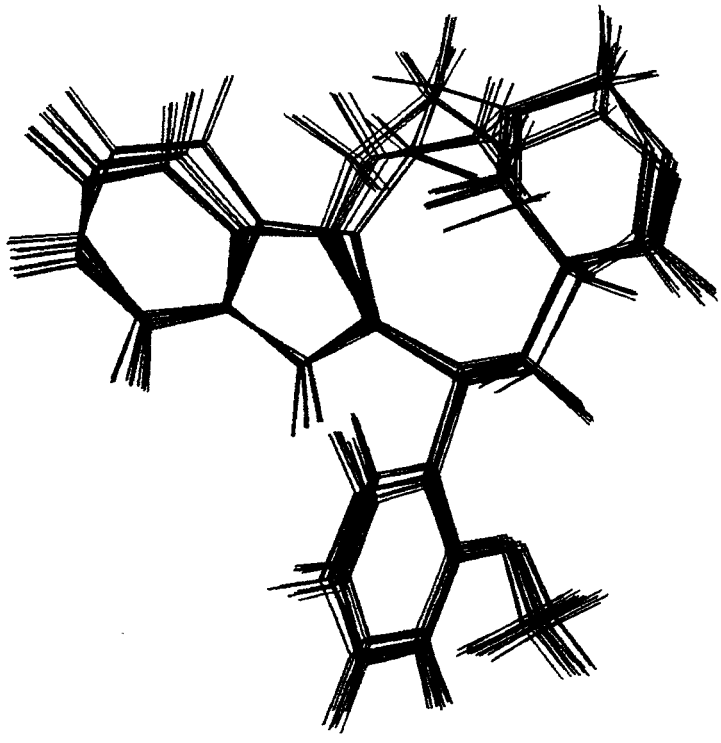


Figure 6. Cluster analysis of the structures generated in a Monte Carlo search of the cleavamine subunit substituted at the 18'α-position by an *o*-methoxyphenyl substituent. The clustered structures show that there are three preferred groups of conformations of the dehydropiperidine ring and the region of the nine-membered azanonine ring comprised of the 19'-, 6'-, 7'-, and 8'-positions. The motional freedom confirmed by this modeling study may explain the considerable broadening of the H5', H19', H7' and H8' protons in the reference spectrum of **1** shown in Figure 1. (Note: The 4' ethyl and the 18' methyl ester groups were removed from the structures after the conformational analysis for clarity in the figure.)

3.44 ppm, which Szantay and co-workers [3] had assigned as ~3.4 ppm. We were also able to unequivocally assign the cleavamine aromatic proton resonances from correlations in COSY and ROESY spectra (see Table 3); three of these resonances were unresolved in the data reported by Szantay, *et al.* [3]. Protonated aromatic carbon resonance assignments in this study also confirm those of Szantay *et al.* [3], and are also in agreement with those reported by Kutney *et al.* [4].

Finally, from the expansion of the ROESY spectra of **1** shown in Figure 5, there were pronounced rOe responses correlating the H14' and 16'-NH resonances at 7.15 and 8.04 ppm, respectively, in the cleavamine portion of the molecule with the 21-methyl triplet of the 20-/21-ethyl group in the vindoline subunit, which resonates at 0.78 ppm. These responses suggest a preferred conformation of the cleavamine subunit relative to the vindoline subunit which must bring these resonances into relatively close proximity, *i.e.* <3.5 Å.

Returning to the problem of broadening in the proton

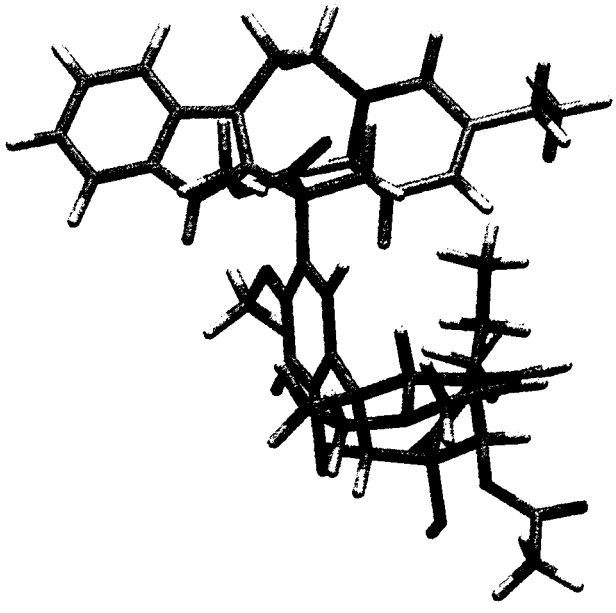


Figure 7. Global energy minimal structure (*in vacuo*) of 3',4'-anhydrovinblastine (**1**) obtained from a Monte Carlo search for 2,000 structures. The orientation of the 16-methoxy group beneath the indole portion of the cleavamine subunit is inconsistent with the results obtained in the ROESY experiments performed on **1**. The dihedral angle formed by C17'-C18'-C15-C16 is +39° which is also inconsistent with the solution conformations of vinblastine reported at -140° [5] and -160° [6].

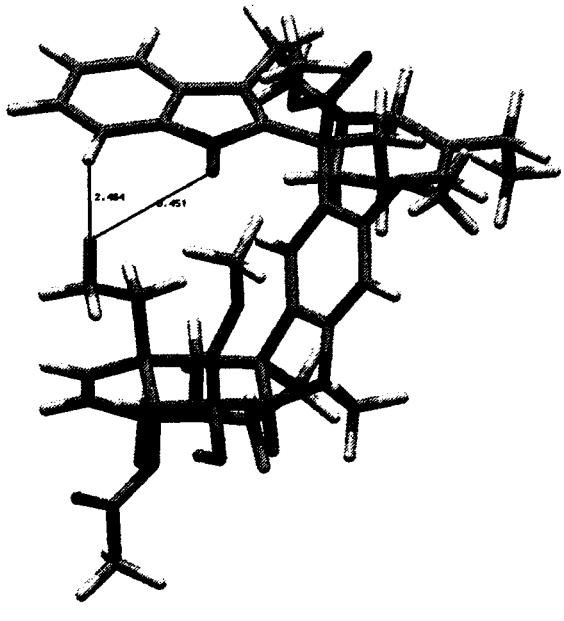
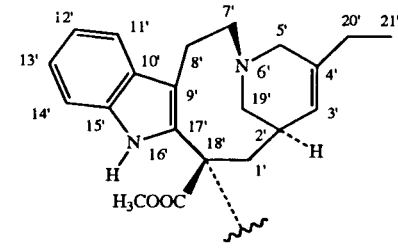


Figure 8. Molecular structure of 3',4'-anhydrovinblastine (**1**) from the Monte Carlo search which is ~0.2 kJ higher in energy than the global minimum energy structure shown in Figure 7. The orientation of the vindoline ethyl group (20/21) beneath the indole portion of the cleavamine subunit is consistent with the results of the ROESY experiment (see Figures 3 and 5). The dihedral angle defined by C17'-C18'-C15-C16 was -153° which is consistent with the results for vinblastine reported in previous studies [5,6].

Table 4

Carbon Resonance Assignments for the Cleavamine "Half" of 3',4'-Anhydrovinblastine (**1**) in Deuteriochloroform



Position		Chemical Shift (δ ppm)	
Biogenetic	IUPAC	Szantay *et al.*[3]	Present Report
17'	1'	34.3	33.5
14'	2'	32.9	30.1
15'	3'	123.8	123.4
20'	4'	139.9	135.0 [a]
21'	5'	52.1	52.0
5'	7'	54.5	53.6
6'	8'	25.7	27.3
7'	9'	117.3	(b)
8'	10'	129.4	128.1
9'	11'	118.3	117.4
10'	12'	122.2	122.7
11'	13'	118.4	119.4
12'	14'	110.5	110.4
13'	15'	135.0	134.3
2'	17'	130.9	129.2
16'	18'	55.4	54.5
3'	19'	45.8	44.6
19'	20'	27.8	27.3
18'	21'	12.3	11.0
16'-$COOCH_3$	18'-$COOCH_3$	174.7	171.0
16'-$COOCH_3$	18'-$COOCH_3$	53.3	52.8

[a] No signal was observed in the carbon reference spectrum for this position. This assignment was made on the basis of a correlation from the 21'-methyl resonance in the HMBC spectrum. [b] No resonance could be reliably assigned for this position. No correlations were observed in the HMBC spectrum from the 16'-NH, the 7'-, or 8'-methylene resonances or the 11' aromatic resonance.

spectrum of resonances in and/or flanking the azanonine ring, we elected to first model the cleavamine portion of the molecule. Our modeling studies were carried out using MacroModel, with the *o*-methoxyphenyl substituent attached at the 18'α-position *in lieu* of the vindoline subunit as above (see also Figure 4). A Monte Carlo search for 1,000 unique structures was performed, after which the structures were subjected to cluster analysis. The results of this study are shown in Figure 6. It should be noted that there are three distinctly different families of conformations for the dehydropiperidine and the portion of the azanonine ring defined by the 19'-, 6'-, 7'-, and 8'-positions. The 7'- and 8'-methylenes have the greatest range of motion which is consistent with the broadening of these resonances in the proton spectrum (see resonance

assignments in Table 3 and the corresponding region of Figure 1).

In modeling the full structure of 3',4'-anhydrovinblastine (**1**), 2,000 structures were created by a Monte Carlo search. The global energy minimal structure is shown in Figure 7. It will be quickly noted that the minimal energy structure brings the 16-methoxyl group into close proximity to the H14' and 16'-NH resonances but that the 20-/21-ethyl group is quite a distance from the cleavamine protons to which rOes were observed. Furthermore, the dihedral angle defined by C17'-C18'-C15-C16 was +39°, which is in sharp contrast to the dihedral angles of 140° reported for vinblastine in solution by Hunter *et al.* [5] and 160° by Gaggelli and co-workers [6]. The second structure found in the Monte Carlo search, 0.2 kJ higher in energy than the global minimum, brought the 20/21-ethyl group of the vindoline subunit into close proximity with the H14' and 16'-NH resonances to which rOes were observed in the expansion of the ROESY spectrum shown in Figure 5. The second lowest energy structure is shown in Figure 8.

EXPERIMENTAL

Purification and Isolation of 3',4'-Anhydrovinblastine.

Cold (3°) deionized water (30 ml) and 3',4'-anhydrovinblastine sulfate (0.05 g) were placed in a 60 ml separatory funnel and shaken until complete solution was obtained. The *p*H was adjusted to 10 with 1*N* sodium hydroxide and the resulting suspension extracted with cold (3°) chloroform-d (2 x 5 ml). The combined extracts were dried at 3° over anhydrous sodium sulfate. The mixture was filtered and the volatiles evaporated under reduced pressure. Atmospheric pressure was restored using nitrogen. The residue was dissolved in 1.6 ml chloroform-d (99.8% d). The solution was capped under nitrogen and an aliquot was taken to prepare the nmr sample used for data acquisition.

Modeling Procedure for 3',4'-Anhydrovinblastine.

Structures were built in MacroModel 4.0 [9] and minimized with the MM3* force field in MacroModel, which is derived from the MM3 force field [10]. Structures were then subjected to Monte Carlo global conformational searching using the default MCMM (Monte Carlo Multiple Minimum) setup in MacroModel. Monte Carlo Multiple Minimum automatically does the following: defines chiral centers for constraint; identifies rotatable bonds for Monte Carlo scanning; selects the number of rotatable bonds which can be varied during a Monte Carlo step; minimizes each Monte Carlo candidate; manages the population of low energy conformations within a 0-50 kJ window; and selects a starting conformation for the next Monte Carlo step from this population on the basis of least usage. The conformational searching of cleavamine and vindoline subunits was also automatically set up by MacroModel. The Truncated Newton (TNCG) minimization method was chosen to achieve good convergence in minimization.

Conformational analysis of the cleavamine portion was initiated to examine the interproton distances in the azanonine ring. A Monte Carlo search generated 1,000 structures; the results indicated this to be a sufficient number of structures to complete the search since each output structure was generated multiple times.

Conformational analysis of the entire 3',4'-anhydrovinblastine (**1**) molecular structure was performed to examine conformational freedom of the cleavamine subunit relative to the vindoline subunit. In particular, this portion of the modeling study was undertaken to examine structures for close intramolecular contacts between protons in the cleavamine and vindoline subunits which could generate strong nOe or rOe responses in a NOESY or ROESY spectrum, respectively. The results indicated that sufficient sampling had taken place for this phase of the modeling study.

Cluster analysis of the Monte Carlo output was performed with version 1.0 of the XCluster module of MacroModel 4.0 [11]. The Arms option was used, meaning that XCluster does rigid-body superimpositions between all pairs of output structures and computes RMS differences on the basis of the atoms. The RMS difference describes the difference or "distance" between conformers. Only the heavy atoms were used in the superimposition and RMS calculation. The Monte Carlo conformations were then clustered on the basis of their RMS differences. Figure 6 contains the superimposed members of one cluster which conveniently illustrate the flexibility in the dehydropiperidine and azanonine rings.

NMR Spectroscopy.

All of the nmr spectra recorded in this study were acquired using a sample consisting of 15 mg of **1** dissolved in 0.6 ml of prechilled (-10°) deuteriochloroform (Cambridge); the probe temperature was maintained at -10° throughout the nmr study of the molecule. The COSY spectrum (Figure 2A) was acquired 2048 x 768 files with 8 transients/file. The data were processed to 2048 x 2048 points during processing and were symmetrized prior to plotting in the absolute value mode. The phase-sensitive TOCSY spectra of **1** were acquired as 2048 x (384 x 2) hypercomplex files with 8 transients/file. Mixing times of 12 and 24 msec were employed. The data were processed using Gaussian multiplication prior to both Fourier transformations and were zero-filled to 2048 x 2048 points during processing. A total of three ROESY spectra were acquired, two with mixing times of 250 msec, and a third with a mixing time of 400 msec. The B_1 spin-lock field employed was 3.2 KHz for one of the 250 msec experiments and the 400 msec experiment. The second 250 msec mixing time experiment utilized a B_1 spin-locking field of 2.2 KHz. All of the ROESY spectra were acquired as 2048 x (384 x 2) hypercomplex files with 16 transients/file. The ROESY data were subjected to Gaussian multiplication prior to both Fourier transformations and were zero-filled to 2048 x 2048 points during processing. None of the ROESY spectra were symmetrized prior to plotting.

Heteronuclear shift correlations were established using the HMQC experiment of Bax and Subramanian [12]. The data were acquired as 1792 x (140 x 2) hypercomplex files; 16 transients were accumulated/file. The experiment was optimized for an assumed average $^1J_{CH}$ = 140 Hz with a 0.4 sec null interval. Spectral widths were 3959 Hz in F_2, and 18227 Hz in F_1 giving acquisition times of 0.226 sec and 0.0077 sec in F_2 and F_1, respectively. The data were linear predicted to 192 files in t_1 and processed to 2048 x 1024 points using Gaussian multiplication

prior to the first Fourier transformation and cosine multiplication prior to the second. Long-range heteronuclear coupling pathways were established using the HMBC experiment of Bax and Summers [13]. The HMBC data were acquired as 4096 x (160 x 2) hypercomplex files. Spectral widths were 5457 and 21,998 Hz in F_2 and F_1, respectively, giving acquisition times of 0.375 and 0.012 sec, respectively. The data were linear predicted to 224 files in t_1, and processed to 4096 x 512 points using a combination of Gaussian and phase-shifted Gaussian multiplication prior to the first Fourier transformation and cosine multiplication prior to the second. The hypercomplex processing scheme of Bax and Marion [14] was used to process the HMBC data.

REFERENCES AND NOTES

[1] T. D. Spitzer, R. C. Crouch and G. E. Martin, *J. Heterocyclic Chem.*, **29**, 265 (1992).

[2] T. D. Spitzer, D. Reynolds, J. P. Shockcor, R. C. Crouch, R. L. Johnson, R. W. Morrison, Jr. and G. E. Martin, *J. Heterocyclic Chem.*, submitted (1994).

[3] C. Szantay, Jr., M. Balazs, H. Bölcskei and C. Szantay, *Tetrahedron*, **47**, 1265 (1991).

[4] J. P. Kutney, L. S. L. Choi, J. Nakano and H. Tsukamoto, *Heterocycles*, **27**, 1827 (1988).

[5] B. K. Hunter, L. D. Hall and J. K. M. Sanders, *J. Chem. Soc., Perkin Trans. I*, 657 (1983).

[6] E. Gaggelli, G. Valensin, N. J. Stolowich, H. J. Williams and A. I. Scott, *J. Nat. Prod.*, **55**, 285 (1992).

[7] J. W. Concrief and W. N. Lipscomb, *Acta Crystallogr.*, **21**, 322 (1966).

[8] O. Kennard and J. Nachman, unpublished observation cited as ref 19 by B. K. Hunter *et al.*, cited as ref [5] above.

[9] MacroModel V 4.0; F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, C. Caufield, G. Chang, T. Hendrickson and W. C. Still, *J. Comput. Chem.*, **11**, 440 (1990).

[10] N. L. Allinger, Y. H. Yuh and J.-H. Lii, *J. Am. Chem. Soc.*, **111**, 8551, 8566, 8576 (1989).

[11] XCluster User Manual, Version 1.0, MacroModel V 4.0.

[12] A. Bax and S. Subramanian, *J. Magn. Reson.*, **67**, 565 (1986).

[13] A. Bax and M. F. Summer, *J. Am. Chem. Soc.*, **108**, 2093 (1986).

[14] A. Bax and D. Marion, *J. Magn. Reson.*, **78**, 186 (1988).