Structure Elucidation and Spectral Assignment of Analogs of Navelbine® (8'-Noranhydrovinblastine)

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During the development of the bis-indole alkaloid anticancer drug Navelbine® (vinorelbine), several chemical degradants of the drug were isolated and identified. These included 7'-nor-6',9'-secovinorelbine (7',8'-bisnor-6',9'-secoanhydrovinblastine) and 4-deacetyl-8'-vinorelbine (4-deacetyl-8'-noranhydrovinblastine). The elucidation of the structure of 7'-nor-6',9'-secovinorelbine is described; the assignment of the proton and carbon spectra of both compounds is contrasted to the shift assignments of Navelbine.

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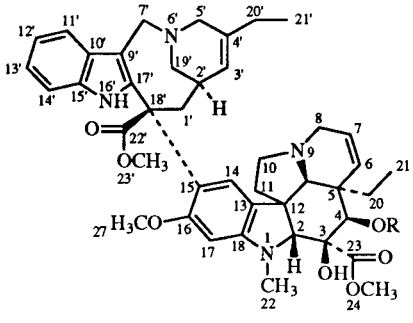
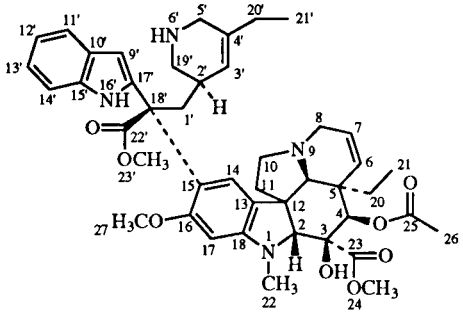
Introduction.

A number of bis-indole alkaloids have become firmly established in anticancer chemotherapy regimens. Examples include compounds such as vincristine and vinblastine [1]. During the course of developing a new bis-indole alkaloid analog, Navelbine® (vinorelbine, **1**, {8'-noranhydrovinblastine}) [2], we had occasion to isolate and identify several chemical degradants of the parent drug. These included 7'-nor-6',9'-vinorelbine (7',8'-bisnor-6',9'-seco-anhydrovinblastine) (**2**) and 4-deacetylvinorelbine (4-deacetyl-8'-noranhydrovinblastine) (**3**). We now report the elucidation of the structure of 7'-nor-6',9'-secovinorelbine (**2**) and the assignment of the ^1H and ^{13}C nmr spectra of both **2** and **3**.



1 (R = -C(=O)CH$_3$)

3 (R = -H)

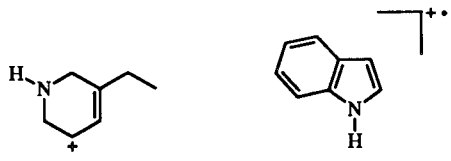


2

7'-nor-6',9'-Secovinorelbine (2).

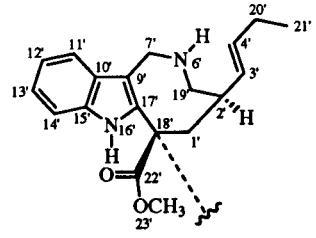
During accelerated decomposition studies of Navelbine® (as vinorelbine ditartrate, 10 mg/ml of aqueous solution under nitrogen, air, or oxygen at 80° for six days), a new degradant of the drug was observed chromatographically. Lyophilization followed by preparative hplc, afforded approximately 250 μg of degradant ultimately identified as 7'-nor-6',9'-secovinorelbine (**2**), a portion of which was used to generate preliminary mass spectral data. The mass spectrum contained a protonated molecular ion at 767 Da, corresponding to a loss of 12 Da relative to Navelbine (**1**). A high resolution mass measurement of the 767 Da ion found in the FAB spectrum gave an accurate mass of 767.4027, corresponding to an elemental composition of $C_{44}H_{55}N_4O_8$ with an error of 0.9 ppm. Thus, this degradant contained one fewer carbon than Navelbine. The ms/ms spectrum of the protonated molecular ion obtained from an ionspray mass spectrum exhibited diagnostically significant ions at 110, 311, and 457 Da. The ions at 311 and 457 Da represent cleavage of the bond between the velbanamine and vindoline portions of the molecule. The 457 Da ion was also observed in the mass spectra of Navelbine and strongly suggests that the vindoline (bottom half) of the molecule was intact. Retrospectively, an ion observed at 323 Da in the mass spectrum of Navelbine has presumably been replaced by the 311 Da ion in the mass spectrum of the degradant, which was consistent with the

loss of a carbon atom from the velbanamine (top half) subunit. The ion at 110 Da was not observed in the mass spectrum of Navelbine but could be accounted for by fragment ion **4** shown below. This observation suggested the possibility that the loss of the carbon atom was possibly in proximity to the ethyl-substituted dehydropiperidine portion of the velbanamine subunit.



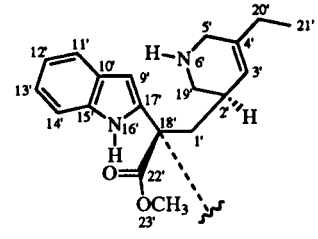
4 **5**

In addition to the 110 Da fragment just discussed, there was also an interesting fragment in the ms/ms spectrum of the doubly charged, doubly protonated ion at 384 Da. That spectrum contained an ion at 117 Da which might be tentatively assigned the indole structure represented by **5**.



6

Extrapolating from the diagnostic fragment ions just described, two plausible modifications of the velbanamine subunit structure might be considered. The first would be a 5'-nor-4',6'-secovinorelbine (**6**); the second would be 7'-nor-6',9'-secovinorelbine (**7**). Of these two structures, the latter was favored since it was consistent with both of the fragment ions, **4** and **5** observed in the ms/ms spectra. The vindoline (lower) subunits of both **6** and **7** have not been shown but would necessarily be intact in both molecules. Two additional structures which would involve the loss of a methylene carbon from the velbanamine subunit, 1'-nor-2',18'-secovinorelbine and 19'-nor-2',6'-secovinorelbine were discounted since neither was consistent with either of the ms/ms fragment ions.



7

Following the completion of the preliminary mass spectral work, the balance of the sample, approximately 200 μg, was dissolved in 160 μl deuterodimethylsulfoxide and transferred to a 3 mm nmr tube. Proton reference, COSY, and HMQC [3], HMQC-TOCSY [4], and HMBC [5] spectra were acquired at 500 MHz using a Varian VXR-500S spectrometer equipped with a Nalorac Z•SPEC® MID-500-3 micro inverse-detection probe. To the best of our knowledge, the research described in this paper represents the first reported usage of micro-detection in the elucidation of the structure of a drug degradant to appear in the literature.

The HMQC spectrum was recorded on a 200 μg sample of the degradant using a micro inverse-detection probe. A singlet in the proton spectrum resonating at 6.17 ppm was correlated directly to a carbon resonating at 101.2 ppm. Both resonances were new and were not contained in the spectrum of the parent drug. The chemical shift of the new proton resonance would be consistent with either the H4'-position of **6** or the H9'-position of **7**. Based on the lack of any multiplet structure to the proton resonance, it was obvious that the correct structure should be assigned as **7**, in which the 6.17 ppm proton resonance could appear as a singlet. In contrast, the H4' resonance of **6** would be coupled to the H20' methylene protons and the H3' vinyl resonance leading, presumably, to a complex multiplet rather than a simple singlet. In comparison, the C9' quaternary carbons of **1** and **3** resonated at 104.8 and 109.0 ppm, respectively.

Another set of changes consistent with the structure was also observed in the shifts of the 5' resonances of **2** relative to **1** and **3**. Thus, consistent with the change from a tertiary to a secondary nitrogen atom at the 6' position, both the proton and carbon resonances are shifted significantly upfield to 3.03/2.99 ppm for ^{1}H and 46.6 ppm for ^{13}C. In contrast, the 5' ^{1}H resonances of **1** and **3** were observed pairwise at 3.90/3.58, 3.76/3.42, and 3.50/3.03 ppm, respectively; the 5' ^{13}C resonances were observed at 51.8, 52.7 and 54.7 ppm, respectively.

Further confirmation of the structure of **2** was also provided by long-range heteronuclear correlations observed in the HMBC spectrum of the molecule. In particular, the H9' proton was long-range coupled to the C10' resonance. Correlations were also observed from the H11' and 16'NH resonances to C9', all of which were fully consistent with **7** as the correct structure for the velbanamine subunit of the molecule.

4-Deacetylvinorelbine (**3**).

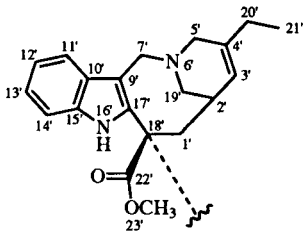
A second, identified degradant of Navelbine, which was

available in quantity from synthetic efforts, was 4-deacetylvinorelbine (4-deacetyl-8'-noranhydrovinblastine) (**3**). Structurally, in view of the relatively small chemical change relative to Navelbine (**1**), the effects on the proton and carbon nmr chemical shifts of **3** were expected to be localized to the vindoline subunit in proximity to the 4-position, and were relatively minor. Total assignment of both the proton and carbon nmr spectra was again accomplished through the concerted interpretation of the COSY, HMQC, HMQC-TOCSY and HMBC spectra, which allowed legitimate comparisons to be made between the assigned spectra of **1** and **3**.

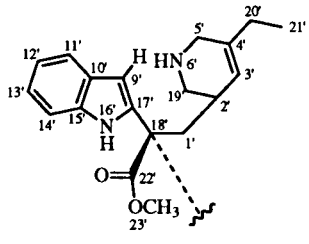
Somewhat surprisingly based on the assigned spectra, deacetylation had virtually no effect on the ^{13}C shift of C4, which resonated 73.8 ppm in the spectrum of **3** and at 74.0 ppm in the spectrum of Navelbine (**1**). Empirically, the removal of an acetyl substituent would normally be

Table 1

Proton Resonance Assignments for the Velbanamine-Derived Subunits of Navelbine (**1**), 7'-Nor-6',9'-Secovinorelbine (**2**), and 4-Deacetylsecovinorelbine (**3**) in d_6-DMSO at 500 MHz



1, 3



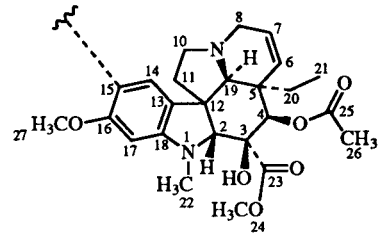
2

Position	Chemical Shift (ppm) Navelbine (1)	2	3
1'	2.86	2.33	2.86
	2.52	2.26	2.49
2'	1.69	2.12	1.58
3'	5.76	4.28	5.72
5'	3.90	3.03	3.76
	3.58	2.99	3.42
6'-NH	—	[a]	—
7'	4.80	—	4.61
9'	—	6.17	—
11'	7.69	7.36	7.76
12'	7.05	6.89	7.04
13'	7.10	6.97	7.11
14'	7.40	7.34	7.43
16'-NH	—	10.57	10.22
19'	3.56	2.92	3.31
	2.69	2.44	2.53
20'	2.02	1.65	1.98
21'	1.02	0.67	1.00
23'	3.65	3.68	3.64

[a] Although the presence of this proton could be demonstrated mass spectroscopically through deuteron exchange from d_8-glycerol, this resonance could not be unequivocally located in the proton nmr spectrum.

Table 2

Proton Resonance Assignments for the Vindoline Subunits of Navelbine (**1**), 7'-Nor-6',9'-Secovinorelbine (**2**), and 4-Deacetylsecovinorelbine (**3**) in d_6-DMSO at 500 MHz



1, 2, and 3 [a]

Position	Chemical Shift (ppm) Navelbine (1)	2	3
2	3.53	3.52	3.44
3-OH	8.64	10.80	—
4	5.05	5.23	3.63
4-OH	—	—	4.61
6	5.24	5.18	5.50
7	5.76	5.78	5.67
8	3.21	3.30	3.18
	2.62	2.73	2.60
10	3.12	3.19	3.11
	2.27	2.48	2.26
11	2.01	2.03	1.96
	1.61	1.93	1.46
14	6.22	6.77	6.27
16-OMe (27)	3.79	3.68	3.76
17	6.40	6.34	6.11
19	2.55	2.65	2.43
20	1.46	1.46	1.52
	1.31	1.23	1.20
21	0.54	0.48	0.62
22-NMe	2.65	2.62	2.67
24-OMe	3.62	3.62	3.65
26-Me	1.94	1.94	—

[a] The acetyl group (25/26) has been removed from **3**.

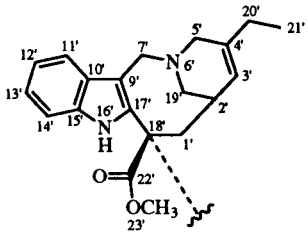
expected to be accompanied by an upfield shift of about 4 ppm at the carbon bearing the hydroxyl remains after cleavage of the ester [6]. In similar fashion, β to the site of deacetylation a downfield shift of about 3 ppm would be expected. However, rather than a downfield shift on deacetylation upfield shifts were observed at both the 3- (85.2 → 80.4 for **1** → **3**) and 5-positions (46.6 → 42.0 for **1** → **3**). These observations suggested that steric rather than electronic effects might play more of a role in affecting carbon chemical shifts in the crowded vindoline subunit. Proton chemical shift behavior was more consistent with the chemistry involved, the H4 resonance shifting upfield from 5.05 ppm in the case of Navelbine to 3.63 ppm in the proton spectrum of **3**.

Conclusion.

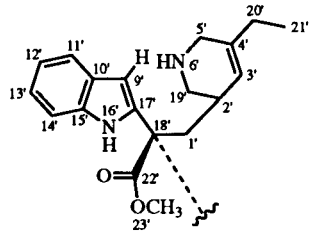
In conclusion, structures can be determined and

Table 3

Carbon Resonance Assignments for the Velbanamine-Derived Subunits of Navelbine (1), 7'-Nor-6',9'-Secovinorelbine (2), and 4-Deacetylsecovinorelbine (3) in d_6-DMSO at 125 MHz



1, 3



2

Position	Chemical Shift (ppm) Navelbine (1)	2	3
1'	35.1	38.2	35.8
2'	29.6	30.5	28.3
3'	122.5	122.9	122.9
4'	131.9	136.0	135.0
5'	51.8	46.6	52.7
7'	46.3	—	46.9
9'	104.8	101.2	109.0
10'	128.9	127.1	128.0
11'	118.1	119.4	118.0
12'	119.5	118.7	119.2
13'	122.2	120.6	122.0
14'	112.1	111.8	112.2
15'	135.1	136.3	135.3 [a]
17'	135.9	139.1	135.2 [a]
18'	54.6	52.5	53.6
19'	44.4	48.5	43.8
20'	26.7	26.5	27.0
21'	11.7	11.4	12.0
22'	173.5	171.3	173.8
23'	52.8	51.7	51.6

[a] Assignments may be transposed.

unequivocal proton and carbon resonance assignments can be derived for molecules as large as the bisindole alkaloid Navelbine on submicromole quantities of material through the use of micro detection techniques. The acquisition of ^{13}C reference spectra can now be determined using either micro detection or recently available heteronuclear Nano-probes™ [7,8] making it plausible to extend these studies to the identification of metabolites using samples in the range of 0.1 micromole [9].

EXPERIMENTAL

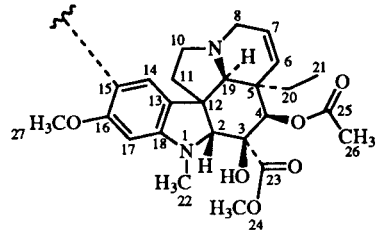
Samples of **1** and **3** used in this study were prepared synthetically. The sample of **2** was isolated from an accelerated degradation study of **1** as the ditartrate salt in aqueous solution at 80° for six days.

Fast atom bombardment mass spectrometry (FABMS) was performed using a VG 70SQ (VG Analytical, Ltd., Manchester, UK) equipped with a cesium ion FAB source. The accelerating voltage was 7 KV, and mass spectra were acquired over the mass range of 100–1500 Da at a resolution of 1500. High resolution mass measurements were made at 8,000 resolution using the $(M+H)^+$ ions of polyethylene glycol (PEG) (Aldrich Chemical Co., Milwaukee, WI) as reference mass standards. The mass spectra were recorded using a VG 11-250J Data System, and the data were processed on a Kratos Mach 3 data system (Kratos Analytical, Manchester, UK).

Ion Spray mass spectra were obtained on a Sciex API III mass spectrometer (Perkin Elmer Sciex, Toronto, Canada) operating in the positive ion mode. The samples were infused using a Harvard Apparatus Syringe Infusion Pump at 20 ml/minute. The orifice plate, discharge needle, and interface plate were set to 50 V, 3.0 mA, and 650 V, respectively. Daughter ion mass

Table 4

Carbon Resonance Assignments for the Vindoline-Derived Subunits of Navelbine (1), 7'-Nor-6',9'-Secovinorelbine (2), and 4-Deacetylsecovinorelbine (3) in d_6-DMSO at 125 MHz



1, 2, and 3 [a]

Position	Chemical Shift (ppm) Navelbine (1)	2	3
2	81.7	82.6	81.5
3	85.2	79.3	80.9
4	74.0	76.0	73.8
5	46.6	42.3	42.0
6	130.3	130.2	131.6
7	124.0	124.2	122.9
8	51.8	46.6	50.0
10	52.1	50.0	48.8
11	49.8	44.1	44.1
12	50.2	52.7	53.3
13	128.4	123.5	122.6
14	122.4	122.2	122.2
15	119.6	121.5	120.7
16	157.8	158.1	157.8
17	93.5	94.2	93.1
18	150.6	152.6	152.3
19	64.3	64.7	63.9
20	30.6	30.5	31.2
21	8.6	7.7	7.9
22	36.9	38.2	37.2
23	169.5	171.3	172.0
24	52.3	51.5	56.2 [b]
25	169.1	170.1	—
26	20.7	20.9	—
27	56.2	56.0	56.1 [b]

[a] The acetyl group (25/26) has been removed from **3**. [b] Assignments may be transposed.

spectra were obtained at a collision energy of 20 eV using argon at a collision gas thickness (CGT) between 1.10 and 1.20 x 10^{14} molecules/cm^2. Mass spectra were recorded on a Macintosh IIfx data system using Sciex software. All spectra were recorded using a step size of 0.1 Da and summation of the mass spectra.

All of the spectral data reported were acquired using a Varian Unity 500 spectrometer equipped with Nalorac Z•SPEC ^{13}C optimized micro dual (MC-500-3) and micro inverse (MID-500-3) probes. Samples were prepared by dissolving 2 micromoles of **1** and **3** in 150 µl of 99.96% deuterodimethyl sulfoxide (Merck), followed by transfer of the sample to a 3 mm nmr tube (Wilmad), using a flexible Teflon needle. The sample of **2** was prepared by dissolving ~200 µg of **2** in 130 µl of 99.96% deuterodimethyl sulfoxide (Merck). Pulse sequences utilized were those described by Bax and co-workers [3-5]. The HMQC-TOCSY data were acquired with ^{13}C decoupling initiated with beginning of data acquisition to retain the direct responses. Mixing times were 18 msec for all of the HMQC-TOCSY spectra recorded.

REFERENCES AND NOTES

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