Structural Characterization of Components of a Mixture-Characterization of a Facile Oxidation product of Cryptolepinone *in situ*

Chad E. Hadden, Wayne K. Duholke, Jane E. Guido, Russell H. Robins, and Gary E. Martin*

Pharmacia & Upjohn, Pharmaceutical Development, Rapid Structure Characterization Group,
Kalamazoo, MI 49001-0199

Maged H. M. Sharaf

Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy
Al-Azhar University, Nasr City, Cairo, Egypt

Paul L. Schiff, Jr.

Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261
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The nmr analysis of a mixture of cryptolepinone and it's 5(*N*)-oxide in a 1.5:1 ratio is discussed. The differentiation of the resonances of the two similar compounds, and elucidation of the unknown 5(*N*)-oxide relied primarily on IDR-GHSQC-TOCSY data in conjunction with ROESY, GHSQC, and GHMBC experiments.

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Introduction.

The nmr data for analysis of any compound is preferably acquired on a pure sample. The assignment of the various resonances is simplified by the knowledge that all resonances correspond to the desired compound. To this end, a considerable effort is applied to the isolation and purification of samples for nmr analysis. Sample purification consumes considerable time and money; resources that are intensely valuable to a researcher.

The ability to accomplish the nmr analysis and structural identification of the components of a mixture (*i.e.* prior to separation) would be undeniably worthwhile. The identification of components of combinatorial mixtures would be one obvious application. Combinatorial research generates a number of similar products in small quantities and in mixtures. Physical separation becomes time consuming and costly. The *in situ* nmr analysis of the combinatorial mixture would circumvent isolation time and effort.

There have been several recent reports detailing the nmr analysis of mixtures. Lin and Shapiro [1] have discussed the application of the GHMBC and homonuclear TOCSY experiments to the verification of similar ester structures; Johnson, *et al* [2], have detailed the applications of the homonuclear TOCSY experiment toward the identification of inositol sugars. However, we have found instances in which the homonuclear TOCSY/COSY experiments are hindered or even obviated by spectral overlap. Methodology to circumvent the shortcomings of the homonuclear correlation experiments would thus be highly desirable, especially when dealing with mixtures.

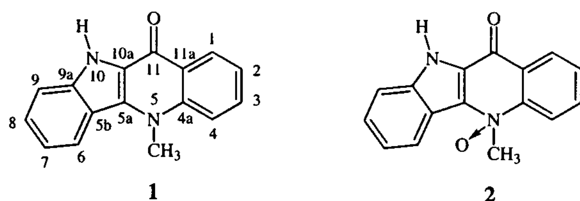
We now report the elucidation of the compounds in a mixture comprised of cryptolepinone and it's 5(*N*)-oxide (1.5:1) using primarily the IDR-GHSQC-TOCSY [3] experiment, supplemented with data from the ROESY, GHSQC, and GHMBC experiments.

Results and Discussion.

A sample of cryptolepinone was stored for a protracted period of time, during which the sample oxidized to a mixture of the alkaloid and the corresponding 5(*N*)-oxide in a 1.5:1 ratio. The aromatic region expansion of the ^{1}H nmr spectrum of a sample of DMSO-degraded cryptolepinone (**1**) is shown as the ^{1}H spectrum plotted above the GHSQC contour plot in Figure 1. As will be readily noted from even a cursory inspection of the ^{1}H spectrum, the sample is comprised of two main components in a ~1.5:1 ratio. There are, in addition, several smaller components contained in the sample, the largest at levels estimated to be roughly 6%, based on integration. No effort was made to characterize these components; they were expected to be out of reach *via* nmr under most circumstances with the exception of submicro probe technology [4].

Aside from the obviously high level of congestion in the aromatic region of the proton spectrum, there are two singlets observed downfield and separated from the aromatic region that are expected to be the N(10)H resonances of the two major components contained in the sample. Based on chemical shift expectations [5], the ^{1}H singlet resonating further downfield was assumed to be cryptolepinone, and was the major component of the mixture. The NH proton singlet just upfield was expected to arise from an oxidized degradation product.

The nmr analysis of mixtures relies predominantly on the use of heteronuclear shift correlation methods. The interpretation of COSY spectra alone is hindered by overlapping peaks and convoluted connectivity pathways; a situation only somewhat ameliorated by homonuclear TOCSY. Hence, it is preferable to employ one of the hyphenated, heteronuclear experimental variants, *e.g.* GHXQC-TOCSY (where X = M or S), to determine proton-proton connectivities in the second frequency domain sorted by the ^{13}C



chemical shift. Specifically, the IDR-GHSQC-TOCSY experiment [6] has several inherent advantages. First, it offers the advantage of inverting the direct response, making it easier to differentiate direct and relayed responses with very similar proton shifts. Additionally, single quantum coherence affords inherently better F_1 resolution than multiple quantum experiments, as evaluated by Reynolds and co-workers [7] comparing the GHSQC and GHMQC experiments, and in a recent study by the authors comparing variants of the GHSQC- and GHMQC-TOCSY experiments that gave similar results [8].

Although it is possible to begin the nmr analysis directly from the acquisition of an IDR-GHSQC-TOCSY spectrum, in which the direct responses are identifiable by virtue of being inverted, it may still be preferable to obtain a conventional GHSQC spectrum when dealing with a mixture of compounds such as that in hand. The subsequent acquisition of an IDR-GHSQC-TOCSY [6] spectrum is expected to facilitate the identification of four separate spin systems, each comprised of four contiguous protonated carbons. At this point, it becomes a task of associating the four-spin systems pairwise, and then orienting them within the carbon skeleton of the two alkaloid components of the mixture. The task of pairwise association and orientation is conveniently accomplished in a single step from the acquisition of a ROESY spectrum. Correlations from the *N*-methyl resonances of the two major components in the mixture will identify the H4 and H6 resonances flanking each of the *N*-methyls, simultaneously orienting the spin systems correctly. Finally, the assignment of the quaternary carbons can be undertaken following the acquisition of a GHMBC [9] spectrum of the mixture.

As the structure of cryptolepinone has been the subject of several recent reports [5,10-13], the details of it's nmr spectral assignment will not be described further. It was, however, fully characterized to preclude any possible misassignment during the elucidation of the *N*-oxide degradant structure. As expected, the shifts of the *N*(5)-oxide are similar to those previously reported for cryptolepinone [5], with a few exceptions, *e.g.* C4a and C5a flanking the oxidized nitrogen. The ^{1}H and ^{13}C shifts, both for cryptolepinone and cryptolepinone *N*-oxide, are presented in Table 1.

The GHSQC spectrum of the mixture (Figure 1) was acquired to establish the one-bond ^{1}H-^{13}C direct correlations. Cursory inspection shows the extensive F_2 overlap of three resonances at about 6.97 ppm. This chemical shift discrepancy is removed in the IDR-GHSQC-TOCSY spectrum, shown in Figure 2. The direct responses are inverted and are shown in red; the relayed responses are shown in black. Hence, a trace can be drawn from the red direct response, through the black cross correlation, to the red

Table 1

^{1}H and ^{13}C chemical shifts (ppm) for the mixture of cryptolepinone (**1**) and cryptolepinone 5(*N*)-oxide (**2**), compared to the previously reported chemical shifts of cryptolepinone [5]. The sample consisted of 5 mg of the mixture in 150 µl dimethyl-d_6 sulfoxide (Isotec). The ^{15}N chemical shifts were obtained from a ^{1}H- ^{15}N GHMBC spectrum at natural abundance [13].

	Cryptolepinone [5]		Cryptolepinone, *in situ*		Cryptolepinone 5(N)-oxide, *in situ*	
Position	^{1}H	^{13}C/^{15}N	^{1}H	^{13}C/^{15}N	^{1}H	^{13}C/^{15}N
1	8.43	126.0	8.46	125.7	7.47	125.0
2	7.36	121.0	7.37	120.8	6.82	118.3
3	7.78	131.9	7.78	131.3	7.61	138.4
4	7.96	116.0	7.97	115.7	7.17	109.9
4a		141.0		140.3		163.2
5		103.4		103.4		188.1
5(N)-CH_3	4.35		4.38		2.78	
5a		131.0		130.6		159.5
5b		116.5		116.6		124.3
6	8.38	123.0	8.39	123.2	6.97	123.9
7	7.19	119.8	7.21	119.3	6.98	122.5
8	7.48	127.0	7.49	127.3	7.32	130.4
9	7.58	113.0	7.59	113.0	6.97	110.8
9a		139.0		139.1		144.2
10		113.1		111.1		134.9
10(N)H	11.95		11.88		10.88	
10a		124.0		123.8		-
11		167.4		166.8		-
11a		123.8		123.5		119.0

direct response of the vicinal neighbor. This process is shown for the quinoline ring system in Figure 2, and the indole ring system in Figure 3.

All three resonances at about 6.97 ppm belong to the same *N*(5)-oxide spin system. The protons resonating at about 6.97 ppm. correspond to two doublets and one triplet resonant pair. This internal triplet spin finally exhibits a correlation to the terminal resonant pair at 6.97/123.9 ppm.

Having correctly sequenced the indolo four-spin system of **2**, there still remains the task of orienting it correctly to the carbon skeleton. The normal method using an rOe correlation from the N-Me resonance to H6 fails in the present

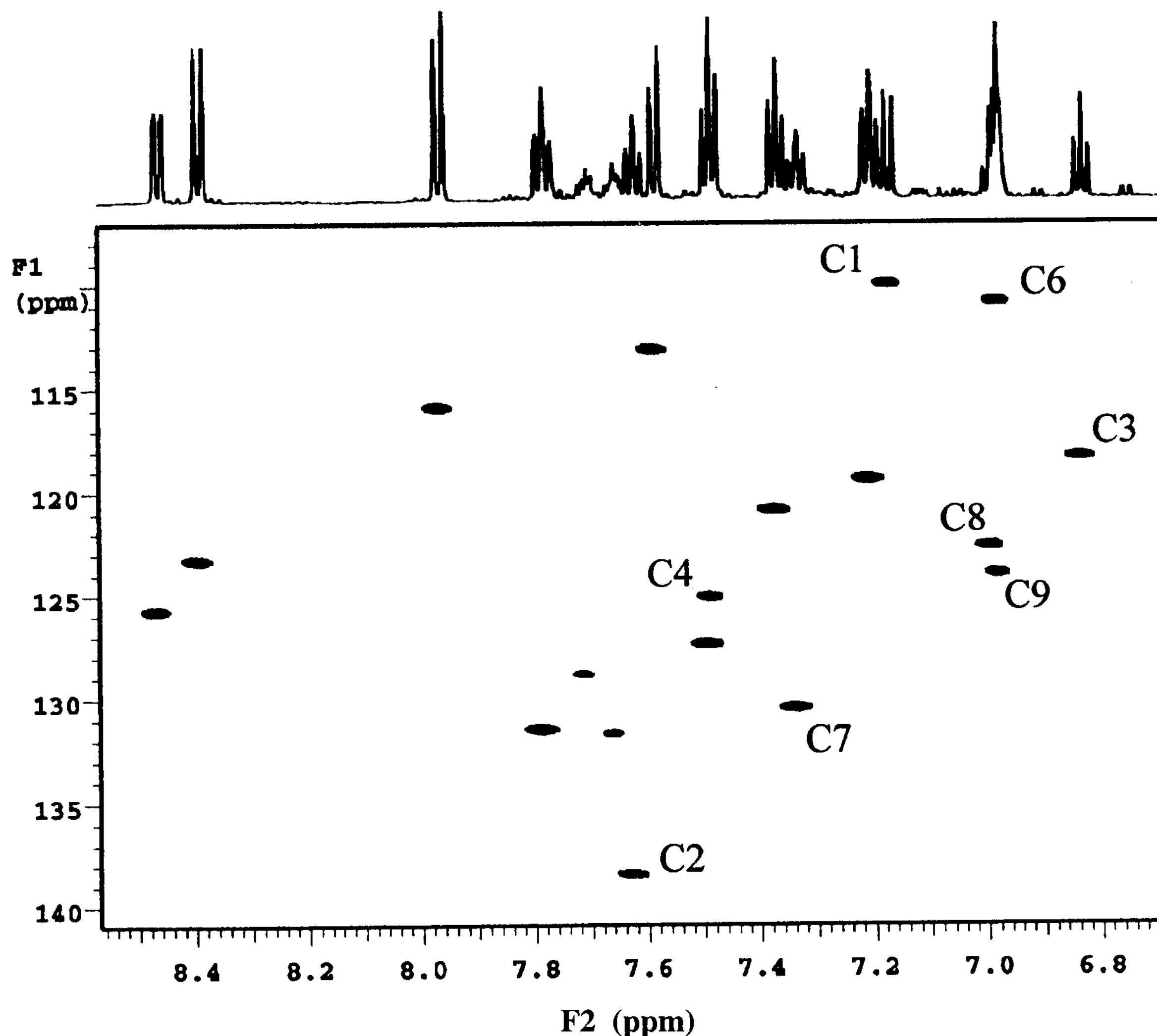


Figure 1. GHSQC spectrum of the mixture of **1** and **2**. The sample consisted of 5 mg of the mixture in 150 µl of dimethyl-d_6 sulfoxide (Isotec). The labeled resonances represent the proton-carbon resonant pairs of the indoloquinoline ring system of **2**. The overlap of the H6, H8, and H9 resonances is partially resoved *via* separation employing the ^{13}C chemical shift, however, the order of spins is unresolved. Data were acquired as 2048 x 256 hypercomplex files in F_1 with 32 transients per t_1 increment. Linear prediction in F_1 afforded a spectrum comprising 2048 x 1024 points prior to transformation. Data were processed using gaussian multiplication prior to both transformations.

of the four-spin system. The first terminal spin pair resonating at 6.97/110.8 ppm exhibits a correlation to the resolved proton triplet resonating at 7.32/130.4 ppm *via* the black relayed response. (The IDR-GHSQC-TOCSY expansion of this spin system is shown in Figure 3.) This proton/carbon pair, in turn, correlates back to the overlapped resonances, specifically to the 6.98/122.5 ppm res- case since H6 and H9 both resonate at 6.97 ppm. Here, we must take recourse to the GHMBC spectrum (not shown). Correlations from the sole resolved proton of the indolo four-spin system at 7.32 ppm and the N-H resonance at 10.88 ppm are observed to a carbon resonating at 144.2 ppm, which can be assigned as C9a. This correlation is unique and establishes the 7.32 ppm resonance as H8.

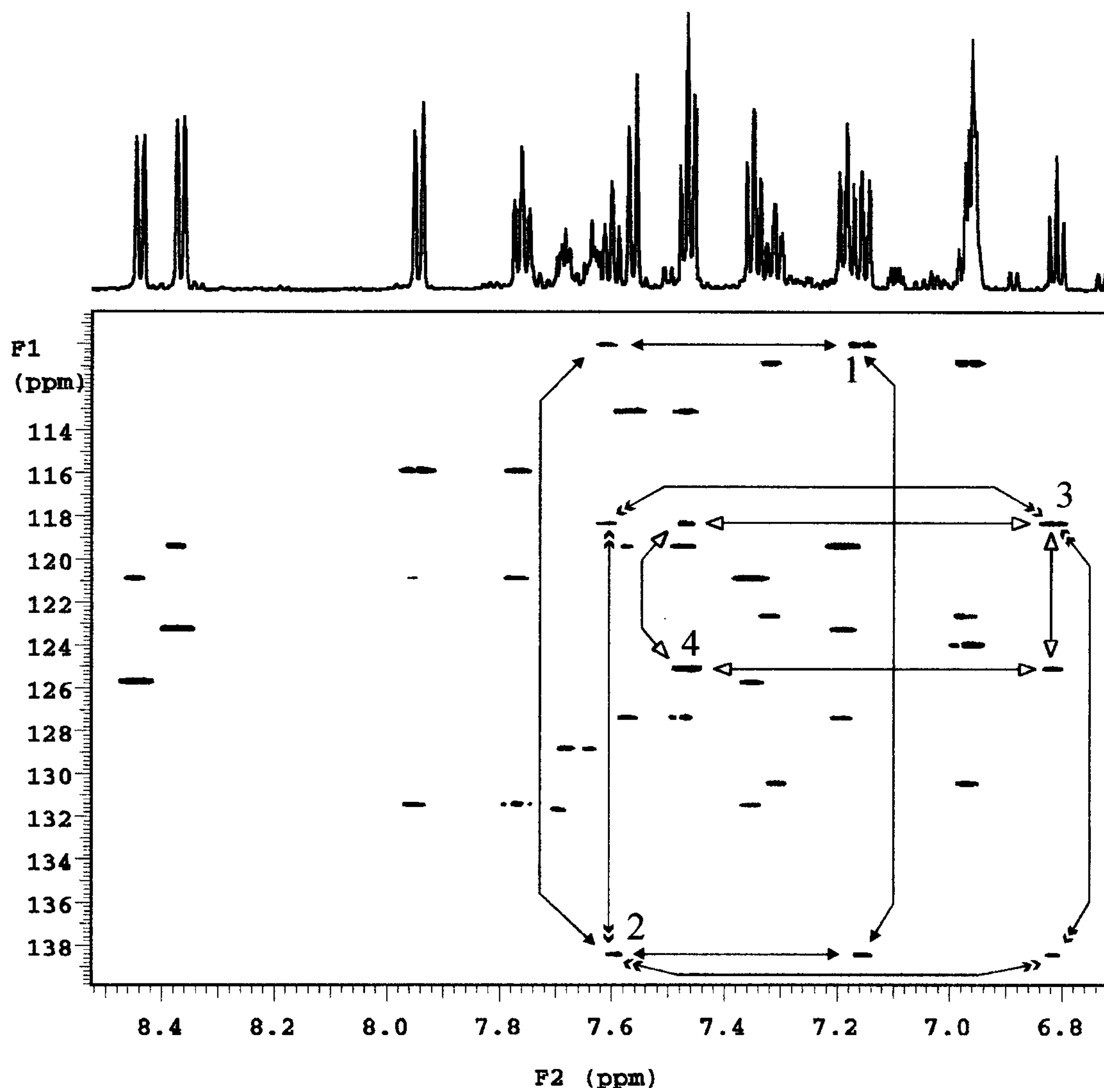


Figure 2. IDR-GHSQC-TOCSY spectrum of 5 mg of a mixture of cryptolepinone (1) and its *N*(5)-oxide in 150 µl of dimethyl-d_6 sulfoxide (Isotec). The experiment was acquired with a mixing time of 24 ms. The inverted direct responses are shown in red, and the relay responses are shown in black. The arrows outline the correlations from the quinoline ring of cryptolepinone *N*(5)-oxide. Correlations from H1 to H2 are outlined with the full arrow heads, H2 to H3 with the barbed arrow heads, and H3 to H4 with the hollow arrow heads. Data were acquired as 4096 x 256 hypercomplex files in F_1 with 32 transients per t_1 increment. Linear prediction in F_1 afforded a spectrum comprising 2048 x 1024 points prior to transformation. Data were processed using gaussian multiplication prior to both transformations.

Hence, 6.97/110.8 ppm resonant pair is assignable as H9/C9, while the 6.97/123.9 ppm resonant pair must be H6/C6. The remaining 6.97/122.5 ppm resonant pair is thus H7/C7, completing the assignment of the indolo four-spin system.

For heavily overlapped spin systems such as this, considerable care must be exercised when selecting a mixing time for the IDR-GHSQC-TOCSY experiment. The mixing time used should be just long enough to afford a vicinal transfer, but no farther. For aromatic systems such as that at hand, a mixing time of 12-24 ms is generally sufficient. Aliphatic systems, which exhibit considerably greater vicinal coupling constant variability, will generally require correspondingly longer mixing times. In addition, it may not be possible to limit magnetization transfer to a single vicinal coupling in these systems. This makes the use of the IDR-GHSQC-TOCSY experiment in the manner described in this report a more challanging, but still manageable task.

The second spin system of the *N*(5)-oxide begins at 7.17/109.9 ppm, and exhibits a correlation to the proton

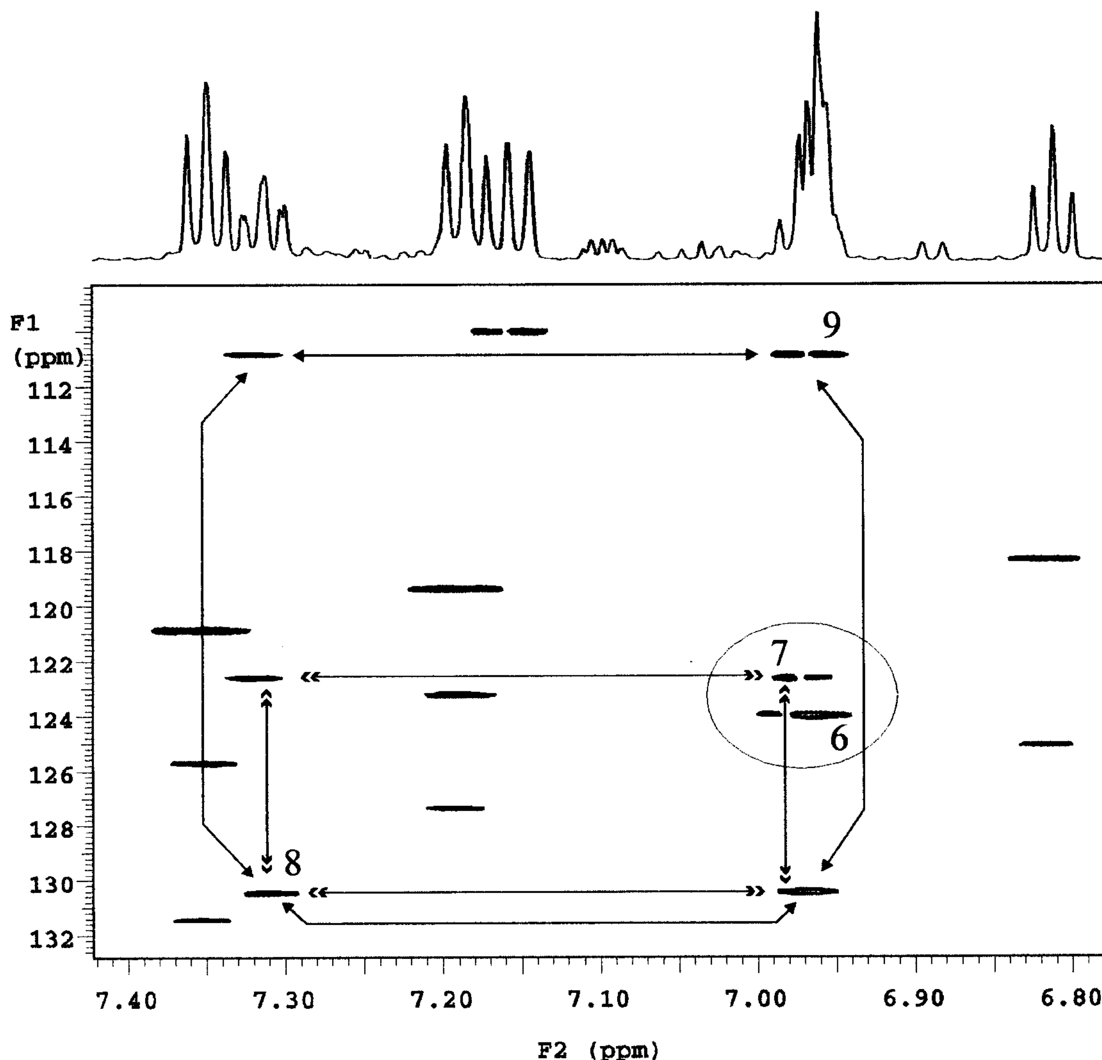


Figure 3. Expansion of the IDR-GHSQC-TOCSY showing the correlations for the indole system of cryptolepinone *N*-(5)-oxide. The overlap of H6, H8, and H9 present the ideal sytem to exhibit the benefits of the IDR-GHSQC-TOCSY experiment, especially with the inverted direct responses shown in red. Correlations from H6 to H7 are contained inside the oval H7 to H8 shown *via* the barbed arrow heads, and H8 to H9 the full arrow heads.

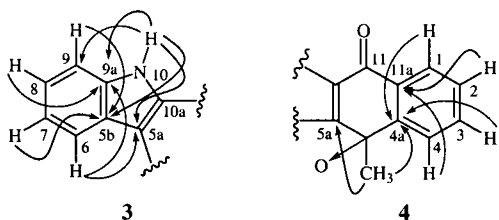
carbon pair at 7.61/138.4 ppm. A correlation to the triplet at 6.82/118.3 ppm connects the second and third resonances, and then finally to the terminal spin at 7.47/125.0 ppm. This latter proton resonance is overlapped with the H8 resonance of cryptolepinone; the responses are still, however, separated by their unique carbon chemical shifts, therein utilizing the full power of the IDR-GHSQC-TOCSY.

The ROESY data provided the correlations to connect the spin systems pairwise in the indoloquinoline ring system. An rOe correlation from the N(10)H resonance at 10.88 ppm to a proton at 6.97 pprn identifies the latter resonance as the H9 resonance. As noted above, correlations from the GHMBC spectrum were alternately employed to orient the indolo four-spin system. A ROESY correlation from the *N*(5)-methyl resonance to the proton resonance at 7.17 ppm, identified that resonance as H4, peri to the *N*(5)-methyl in the quinoline system.

The H2 and H4 resonances at 7.17 and 6.82 ppm, respectively, exhibit correlations to the carbon resonating at 119.0 ppm. This carbon is located *alpha* to the carbonyl of the quinoline ring system at position 11a. The H1 and H3 resonances at 7.47 and 7.61 ppm, respectively, as well as the *N(*5)-methyl protons resonating at 2.78 ppm, exhibit correlations to the carbon resonating at 163.0 ppm, assigning this carbon as the quaternary carbon in the quinoline ring system at position 4a. The *N*(5)-methyl protons, N(10)H, and a proton resonating at 6.97 ppm, which must be H6, all exhibit a correlation to the carbon at 159.5 ppm, identifying it as the C5a resonance. The resonances for C4a and C5a are shifted substantially downfield relative to other members of

this class of indolo[3,2-*b*]quinoline alkaloids [5,10-13]. The cryptolepinone shift for C4a resonates at 141.0 ppm (a difference of +22 ppm); C5a resonates at 131.0 pprn (a diflerence of + 18.5 ppm) [10]. The unique downfield ^{13}C shifts of the *N*-oxide C4a and C5a pinpoint the *N*-oxidation at the N(5) position. This assignment has been independantly confirmed by long-range ^{1}H-^{15}N GHMBC [14].

The H6 and H8 protons at 7.32/130.4 and 6.97/123.9 ppm, respectively, show a correlation to the carbon at 144.2 ppm. The N(10)H resonating at 10.88 ppm also shows a weak two-bond correlation to this carbon, identifying it as C9a. As described above, correlations to C9 were pivotal in the orientation of the indolo four-spin system. The diagnostically important long-range correlations are shown by **3** and **4** as the separate indolyl and quinolinyl systems, respectively.



Preliminary lc/ms (liquid chromatography/mass spectrometry) analysis of the degraded sample gave two major peaks. The earlier eluting peak had a parent ion at m/z 249 da that is consistent with the mass of **1**. The later eluting peak gave a parent ion at m/z = 265 da, consistent with the addition of an oxygen to cryptolepinone. The addition of oxygen is due to the *N*-oxidation of the alkaloid, presumably at the tertiary N(5) position as represented by **2**, which has been confirmed [14]. In addition to the two major peaks, there were a number of much smaller peaks in the chromatogram, suggestive of additional impurities.

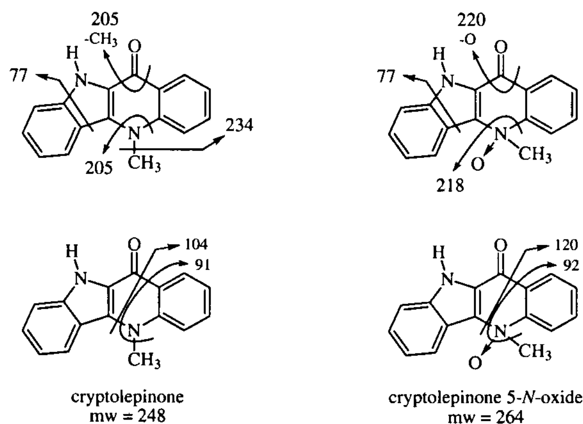


Figure 4. The observed fragmentation of cryptolepinone and cryptolepinone *N*(**5**)-oxide.

The observed fragmentation pathways of cryptolepinone and cryptolepinone *N*(5)-oxide are illustrated in Figure 4. Fragment ions that locate the dative oxygen on N(5) are those at m/z = 218, 120, and 92; the m/z = 120 ion is 16 amu heavier than the corresponding 104 ion in cryptolepinone. Fragment ions that result from the loss of the dative oxygen tend to be within ± 1 amu of the corresponding fragment ions in the parent molecule; in this case, the ions at m/z = 218 and 92 in the *N*-oxide spectrum.

The accurate mass of cryptolepinone *N*(5)-oxide was measured as 264.08940 daltons. The best fit within the valence rules is $C_{16}H_{12}N_2O_2$ differing by 1.8 ppm from the theoretical mass. The mass spectral data support the identification of the dimethyl sulfoxide-induced degradation product of cryptolepinone as cryptolepinone *N*(5)-oxide.

Conclusions.

Utilizing the nmr strategy described in this report, the elucidation of the *N*(5)-oxide degradant, **2**, in the presence of the cryptolepinone parent compound, **1**, is fairly straight-forward. The IDR-GHSQC-TOCSY [6] data played a critical role in the successful assignment of the degenerate indolyl four-spin system. The important downfield shifts of C4a and C5a are consistent with *N*(5)-oxidation. Also consistent with *N*(5)-oxidation, the *N*(5)-methyl proton singlet is shifted upfield by almost 1.6 ppm, due to the altered electronic environment of the *N*-oxide. Interestingly, the N(10)H is shifted upfield by about 1 ppm, consistent with the transmissibility of the electronic effects of the *N*-oxide through the double bond to N(10) [13]. The nitrogen chemical shift changes on *N*-oxidation in this system have been examined (Table 1, [14]) , and there is indeed a significant downfield shift in the N(10) resonance (+22.5 ppm) relative to cryptolepinone (**1**), confirming the transmission of electronic effects between the two nitrogens that was inferred from the ^{1}H data [12]. As expected, N(5) shifted downfield +83.5 ppm to 188 as a consequence of *N*-oxidation [14].

EXPERMENTAL

The nmr sample of **1** was stored in the laboratory as 5 mg in 150 µl, dimethyl-d_6 sulfoxide (Isotec) in a 3 mm nmr tube (Wilmad). After a protracted period of time (~ 36 months), the sample was re-examined and determined to contain an impurity, presumably an oxidation product. All data was acquired on the mixture with no purification.

The lc/ms analysis was done on a Finnigan TSQ-7000 triple quadrupole mass spectrometer. The chromatographic system consisted of a Zorbax Eclipse XDB C8 4.6 x 50 mm column, with the mobile phase consisting of acetonitrile, water, and trifluoroacetic acid, which was run in a linear gradient at a flow rate of 1 ml/minute over 30 minutes, beginning with 95% water, and ending with 5% water. The cryptolepinone *N*(5)-oxide had a RRT of about 1.1 (relative to cryptolepinone). Product ion spectra were obtained for both cryptolepinone and cryptolepinone *N*(5)-oxide over a range of 10 to 275 amu. The collision energy was 50 eV with a collision gas pressure of about 2 torr indicated.

High resolution mass spectral data were obtained on a Finnigan MAT-900ST mass spectrometer operating in the micro-ESI (micro electrospray ionization) mode. Accurate mass measurement was carried out by linear E-scan peak matching at a resolution of 11,600 (m/Δm, 10% valley definition) using reference ions from PEG 200 $(C_{21}H_4O)_5H_2ONa$ and$(C_{21}H_4O)_6$-H_2ONa at 261.13141 and 305.15762 Da to bracket the sample sodiated-molecular ion.

All nmr experiments were performed using a Varian INOVA 600 MHz three channel nmr spectrometer operating at a proton frequency of 599.75 NIHz with 28 channel Oxford shims, and a Nalorac Z•SPEC™ MIDTG-600-3mm micro inverse-detection, triple resonance gradient nmr probe. The pulse sequences used are from the vendor-supplied pulse sequence library, and were employed without modification. Relevant experimental parameters are described in the figure captions.

REFERENCES AND NOTES

* To whom inquiries should be addressed Pharmacia & Upjohn Pharmaceutical Development Rapid Structure Characterization, Group Mail Stop 4821-259-277, 7000 Portage Road Kalamazoo, MI 49001-0199, phone: (616) 833-6283, fax: (616) 833- 6743, e-mail: gary.e.martin@am.pnu.com

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