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Brevetoxin-3: Total Assignment of the ^{1}H and ^{13}C NMR Spectra at the Submicromole Level

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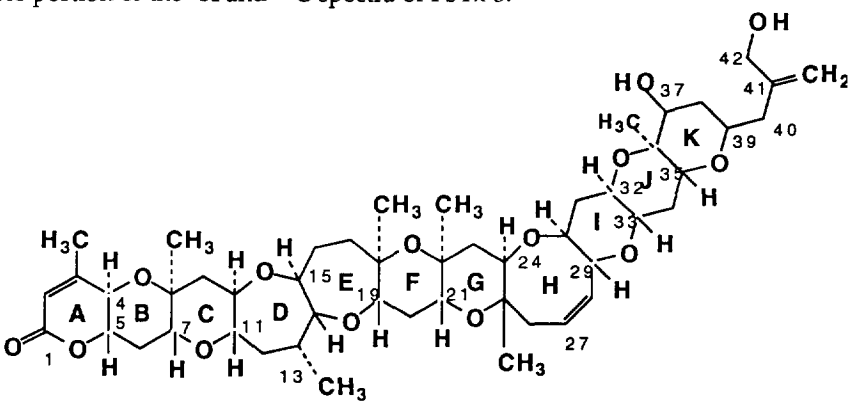
Abstract: The proton and carbon NMR spectra of the marine polyether toxin, brevetoxin-3, are totally assigned using a series of 2D NMR experiments which included: TOCSY, ROESY, HMQC, HMBC, and IDR-(Inverted Direct Response)-HMQC-TOCSY. All work was performed on a sample consisting of 800 μg (0.95 μmole) at 500 MHz.

Toxic events due to red tides have been described by Lasker and Smith[1] and attributed to the dinoflagellate *Gymnodinium breve*. The first such toxin, brevetoxin-B, was identified from a crystal structure as a complex polycyclic ether, and reported in 1981 by Lin et al.[2] Baden[3] has reviewed the pharmacologic properties of the brevetoxins, and summarized the structures known through 1989. Because of the limited available quantities of these materials, their structure elucidation has been severely hampered, largely by the relative insensitivity of the NMR techniques. To date, ^{13}C assignments based on INADEQUATE experiments with labeled materials[4] and ^{13}C-detected HOHAHA[5] have appeared only for brevetoxin-B and -A, respectively. Inverse-detected heteronuclear shift correlation techniques[6] can help considerably, but conventional 5 mm probe technology still necessitates milligram samples. Inverse-detected techniques have not yet been applied to the elucidation of new brevetoxins although they have been applied to the spectral assignment of ciguatoxins (CTX)[7-9], and in the elucidation of the structure of maitotoxin (MTX).[10,11] The relatively recent introduction of micro detection probes[12-14] offers considerable promise in the elucidation of extremely complex natural product structures, e.g. brevetoxins, ciguatoxins, maitotoxins, etc., when small samples are frequently

unavoidable. We report here the total assignment of the ^1H- and ^{13}C nmr spectra of brevetoxin-3 (PbTx-3) (**1**)[15] accomplished using a 0.95 μmole sample dissolved in 120 μl of d_6-benzene.

Acquisition of homonuclear 2D data on submilligram quantities of material, even for compounds as complex as PbTx-3, has been trivial. Homonuclear COSY, TOCSY, and ROESY spectra of PbTx-3 were acquired and served, where ^1H resonances were resolved, to establish proton-proton connectivities, either through bonds or through space, in a manner analogous to that employed with brevetoxin-A.[16] Direct proton-carbon shift correlations were established from an HMQC spectrum. All data shown in Fig. 1, the HMQC[17] spectrum, and both ^1H- and ^{13}C reference spectra, were acquired in <10 h using a Varian Unity 500 equipped with Nalorac Z•SPEC MID-500-3 and MC-500-3 micro detection probes.[18] These data alone, in conjunction with the DEPT, homonuclear COSY, and TOCSY spectra, allowed the assignment of a considerable portion of the ^1H and ^{13}C spectra of PbTx-3.



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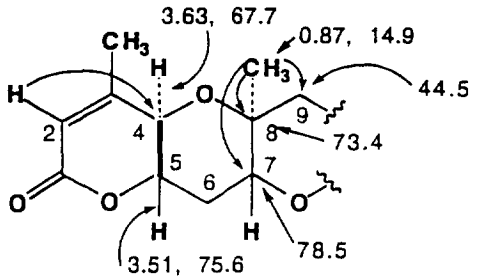

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The balance of the resonance assignments presented in Table 1 were derived from the concerted application and interpretation of the HMQC, HMQC-TOCSY[19,20], and HMBC[21] spectra of **1**. This approach overcame potential ambiguities in ^1H resonance assignments that arose through overlaps in congested regions of the ^1H spectrum. As is shown by structural fragment **2**, a convenient starting point is provided by the H2 vinyl proton. An assignment strategy can then be developed by exploiting a long-range coupling observed in the HMBC spectrum correlating H2 with the C4 methine carbon resonating at 67.7 ppm. The H2-C4 correlation, along with those of the bridgehead methyl groups, C8 and C18, are labeled in the HMBC spectrum of **1** shown in Figure 2.

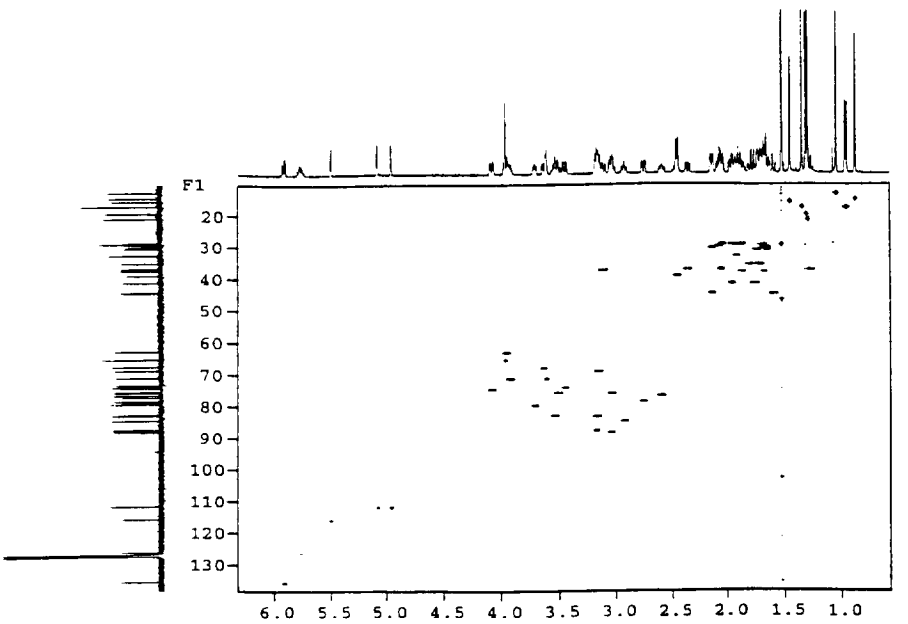


Figure 1. 2D HMQC spectrum acquired with 1. Spectral windows were 3728 Hz in F_2 and 16466 Hz in F_1. Data were obtained as 320 States-TPPI increments with 16 transients/increment and a 1.1 sec recycle time. ^{13}C decoupling was applied with a B_1 of 5 KHz garp modulation. Acquisition times were 206 ms in F_2 and 19 ms in F_1. Data were processed with a 0.095 sec gaussian function before the first FT and a cosine function prior to the second FT.

Table 1. ^1H- and ^{13}C-nmr resonance assignments of brevetoxin-3 (PbTx-3) in benzene-d_6 at 500 MHz.

Position	δ^1H	$\delta^{13}C$	Position	δ^1H	$\delta^{13}C$	Position	δ^1H	$\delta^{13}C$
1	–	161.65	15	3.54	82.75	29	3.71	79.46
2	5.50	115.67	16	1.91, 1.68	29.04	30	3.04	75.78
3	–	158.55	17	1.87, 1.67	37.55	31	2.06, 1.28	36.94
3-Me	1.52	29.14	18	–	77.16	32	3.16	68.82
4	3.63	67.72	18-Me	1.29	21.23	33	2.60	76.58
5	3.51	75.55	19	3.04	88.14	34	2.13, 1.66	30.20
6	2.08, 1.67	29.63	20	2.04, 1.89	28.85	35	3.95	63.01
7	2.76	78.47	21	3.45	73.97	36	–	74.00[a]
8	–	73.37	22	–	73.79[a]	36-Me	1.04	13.14
8-Me	0.87	14.93	22-Me	1.30	19.65	37	3.92	71.08
9	2.14, 1.60	44.50	23	1.96, 1.76	41.24	38	1.74, 1.65	30.74
10	3.17	83.02	24	4.07	74.50	39	3.61	71.15
11	2.93	84.55	25	–	79.14	40	3.12, 2.37	37.07
12	1.80, 1.72	35.30	25-Me	1.34	17.45	41	–	146.87
13	1.91	32.69	26	2.46	39.10	42	3.96	65.30
13-Me	0.95	17.45	27	5.77	126.06	43	5.08, 4.95	111.61
14	3.17	87.49	28	5.91	135.50			

[a] Possibly permutable assignments.

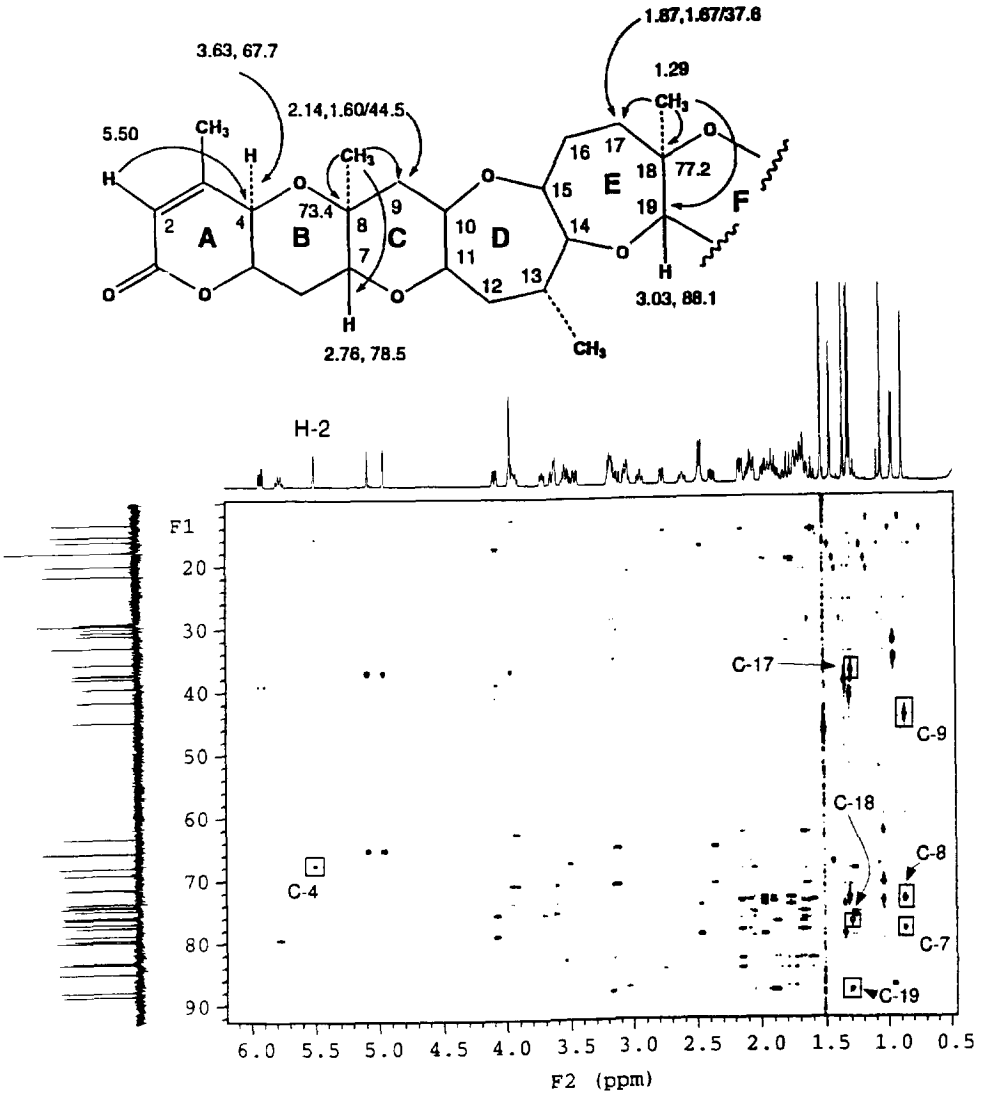


Figure 2. Excerpt from a 2D HMBC spectrum acquired with **1**. Acquisition times were 289 ms in F_2 and 18 ms in F_1 which was extended to 25 ms by linear prediction. Spectral windows were 3540 Hz in F_2 and 19609 Hz in F_1 with 352 States-TPPI increments. Data were processed with a 0.120 sec gaussian function (shifted by 0.76 sec) prior to the first FT; a cosine function was applied before the second FT.

Once the H4/C4 resonant pair are identified, correlations in either the homonuclear TOCSY or HMQC-TOCSY spectra can then be used to establish and expand the proton-proton connectivity network. In particular, we preferred to rely on the IDR-(Inverted Direct Response)-HMQC-TOCSY[20] rather than on the homonuclear TOCSY experiment because of the number of overlapping key resonances in the proton spectrum. For example, once the correlation from the H2 vinyl proton to C4, resonating at 67.7 ppm, is established from the HMBC spectrum, H4 resonance is identified as the proton resonating at 3.63 ppm from the HMQC spectrum. To expand the structural fragment being developed, H4 must next be correlated to the H5 resonance. The latter, H5, resonating at 3.51 ppm, is partially overlapped by the H14 resonance at 3.54 ppm. While there is still a usable difference in chemical shift, insofar as the utilization of homonuclear TOCSY data are concerned, the assignment may be more clearly developed through the use of the IDR-HMQC-TOCSY data. Figure 3 is constructed in color to underscore the clarity and resolution afforded by the IDR-HMQC-TOCSY experiment. All of the oxygenated bridgehead methine to methine connectivities are illustrated in Figure 3; the direct ^{1}H - ^{13}C correlations are denoted as black contours whereas the relayed TOCSY responses are shown as red contours. Each methine pair is also highlighted by a dashed square. Looking elsewhere in this same IDR-HMQC-TOCSY dataset allows a continuation of the construction of the structural fragment begun with the HMBC H2 to C4 connectivity developed earlier. The H6 protons (boxed negative responses at 29.6 ppm in Figure 4), exhibits relayed magnetization to both the H5 and H4 protons, as well as an intense correlation to what must be H7. The fact that the only methylene resonance which can show connectivity to H4 and H5 is C6 leads to this irrevocable assignment. In similar fashion, the structural fragment is expanded to include the H7/C7 methine resonant pair at 2.76/78.5 ppm. Since the 8-position is a quaternary carbon, the connectivity network being developed from the IDR-HMQC-TOCSY data dead ends. To continue the spectral assignment and/or structure elucidation in the case of an unknown, the H7/C7 resonant pair must be linked to either the quaternary carbon or the next protonated center by means of other experimental data.

Returning again to the HMBC spectrum (Figure 2), correlations from the bridgehead methyl group, 8Me, resonating at 0.87 ppm, are observed to C7 (78.5), a quaternary carbon, C8 (73.4), and methylene carbon resonating at 44.5 ppm, which may be assigned as C9. These correlations unequivocally locate the bridgehead methyl singlet at the B/C ring juncture. By bridging the C8 quaternary carbon through the use of correlations from the 8-methyl to the C7 methine, C8 quaternary, and C9 methylene resonances, we are positioned to begin the assignment of the next group of contiguous protonated carbons, C9 through C18, as shown by 3. Through concerted interpretation of correlations in the IDR-HMQC-TOCSY and HMQC spectra, the vicinal proton-proton connectivity network beginning from H9 can be developed and expanded, one methine or methylene at a time.

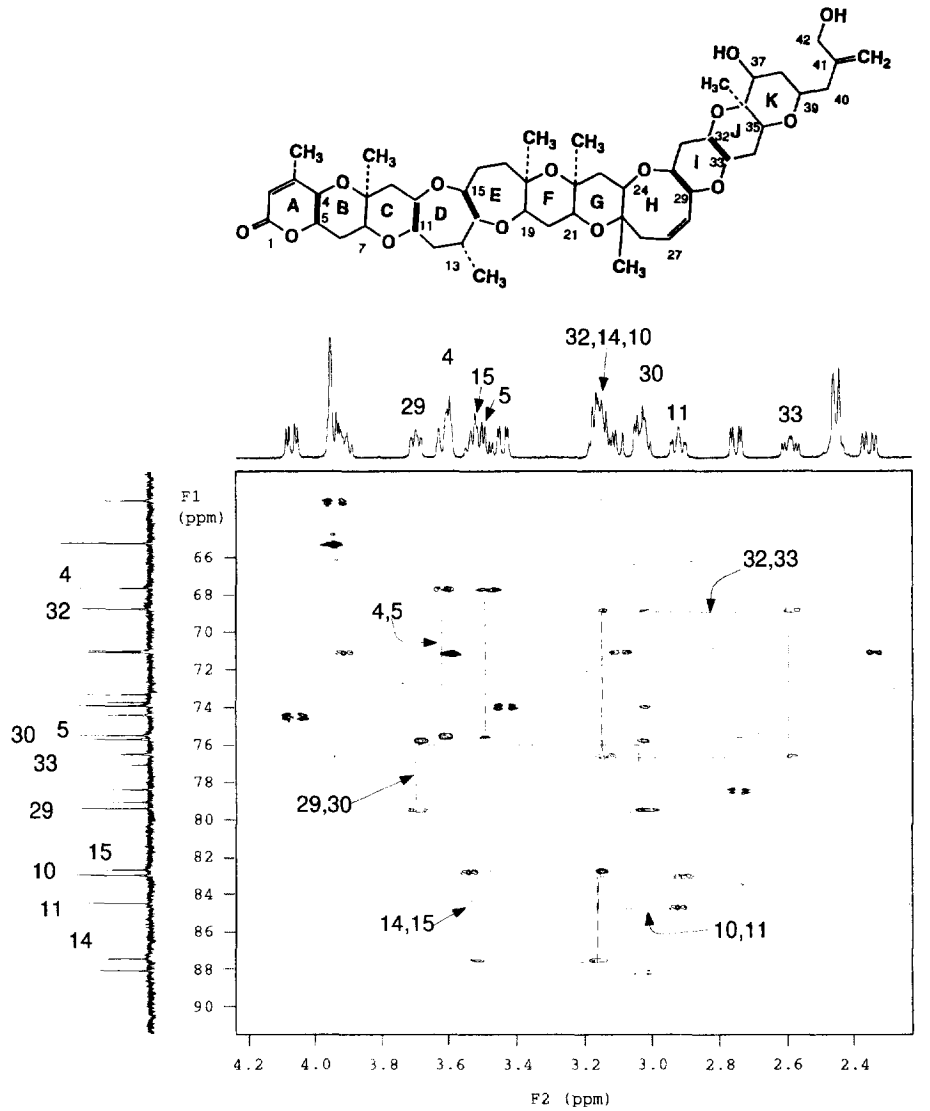
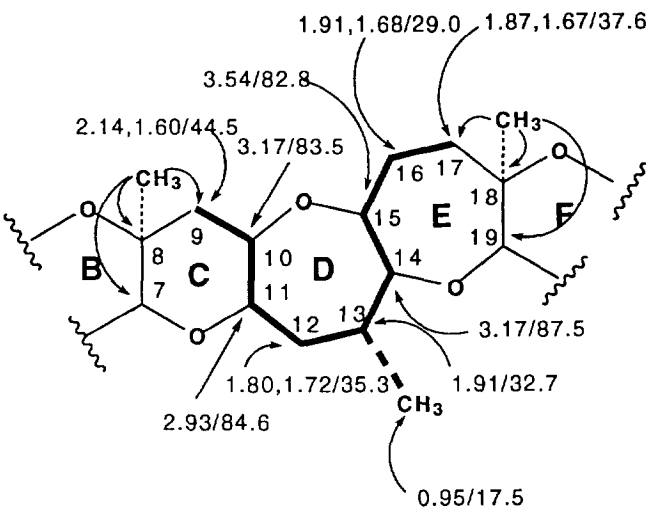


Figure 3. Region from an IDR-HMQC-TOCSY experiment detailing the oxygenated methine to methine correlations. Direct ^{1}H - ^{13}C correlations are shown in black; the TOCSY relayed peaks are in red. Data were acquired as 1152 States-TPPI pairs with spectral windows of 3738 Hz in F_2 and 10558 Hz in F_1 and corresponding acquisition times 206 ms and 109 ms, respectively. The highlighted correlations are noted by dark lines in the accompanying structure.



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In a similar fashion, vicinal connectivity fragments were established for fragments defined by positions 19-21, 23-24, 26-35, and 37-40. Bridgehead methyls located at the 18-, 22-, 25-, and 36-positions served to link the contiguous protonated carbon fragments to one another, and simultaneously assigned the respective methyl-bearing quaternary carbon resonances. Finally, the exo-methyene at the 41-position was used to link the 40-methylene to the terminal hydroxyl-bearing 42-methylene. This series of repetitive operations afford a single, large, structural "fragment", which may be thought of in terms analogous to squalene before cyclization to a steroid. Clearly, there are positions along the backbone of the structural fragment where oxygen atoms must serve as ether bridges to other points along the chain, this contention obviously based on carbon chemical shift considerations.

The portion of the final structural "fragment" comprised of rings A-E is shown in Figure 5. Bonds denoted by dashed lines were established using correlations from the HMBC spectrum. Contiguous protonated carbons denoted by solid, boldface bonds were linked using homonuclear connectivity information from the IDR-HMQC-TOCSY spectrum as is shown in Figures 3 & 4. Solid bonds broken by a wavy line going to oxygen represent positions along the fragment backbone where oxygen must be attached to the backbone based on carbon chemical shifts. At this point, the sole task remaining to be accomplished is the correct linkage of adjacent oxygen-bearing carbons to one another.

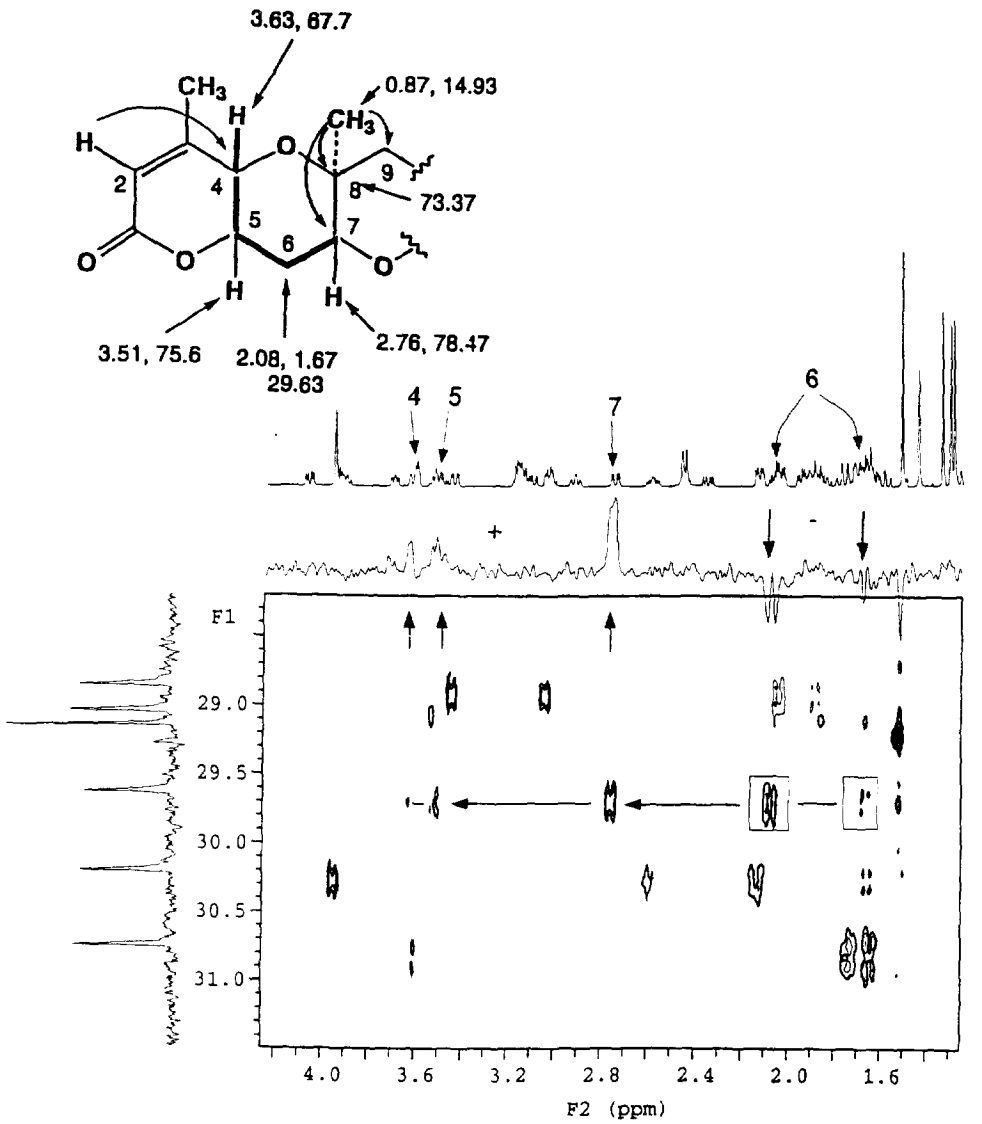

Figure 4. A further expansion from the IDR-HMQC-TOCSY experiment described in Figure 3 illustrating the relayed homonuclear ^{1}H couplings originating from the C6 methylene resonances. Both the F_2 trace through the C6 ^{13}C resonance and the identical region from the ^{1}H spectrum are shown. All key resonances are labeled and noted on the structural fragment.

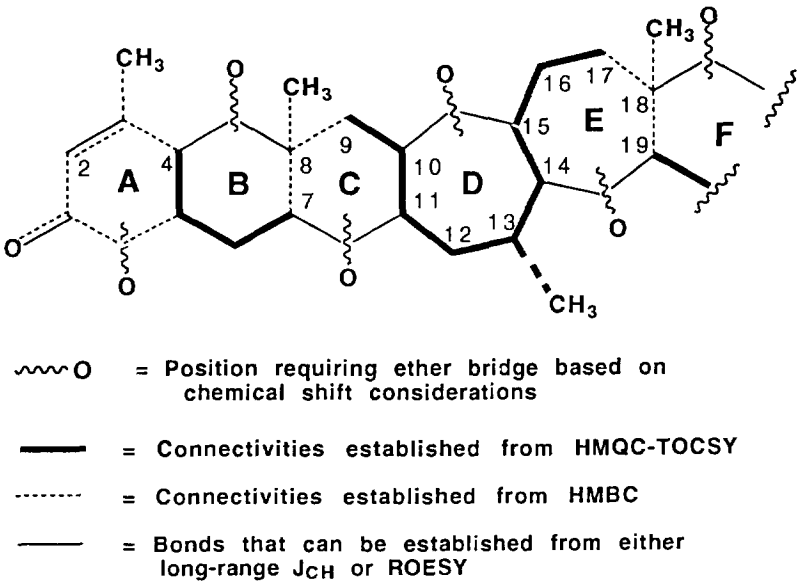

Figure 5. Structural fragment of PbTx-3 represented by rings A-F. The technique used to establish various bonds is given above. No stereochemistry is implied for the methyls at the 3-, 8-, or 18-positions in this figure.

Two alternatives are available for the linkage of non-contiguous oxygen-bearing carbons. First, ether oxygens may be bridged via long-range heteronuclear couplings in the HMBC spectrum where a resolved, protonated methine is available. Alternatively, ROESY connectivities may also be used to link protons attached to either terminus of the ether bridge to one another. The former approach is unequivocal in that it cannot span distances greater than four bonds. If a correlation is observed in the HMBC spectrum, the ether bridge in question will be irrefutably established. Moreover, most ether bridges of **1** offer the possiblity of using two HMBC correlations to establish the ether bridge, affording redundant confirmation or a backup if one of the methines is poorly or completely unresolved. If we were dealing with an unknown structure, ROESY is not necessarily unequivocal. While there are defined limits across which a rOe can be observed, the sole requirement in terms of the molecular framework, is that the two cross-relaxing nuclides must be proximal in space. Consider the following example: an HMBC correlation irrefutably establishes the C10-O-C15 ether bridge via the long-range correlation from H15-C10. The converse pathway, via $^{3}J_{H10C15}$, is equivocal as will be discussed further below. In contrast, in a ROESY spectrum, H10 and H15 could exhibit a rOe between one another. Just as easily, H10 could also exhibit a rOe to H24, the next proton on the "north-side" of the molecule with appropriate stereochemistry, if H10 and H24 were in correct proximity to one another. It is also worth noting that H10 and H14 have exactly the same proton chemical shift making it impossible to use rOe correlations to or from H10 for

assignment purposes without resorting to HMQC-ROESY[22]. For this reason, when working to establish an unknown structure, care must be taken when using rOe or nOe correlations to bridge heteroatoms or quaternary carbons. We prefer to use HMBC and ROESY connectivities, when possible, in a complementary and concerted fashion.

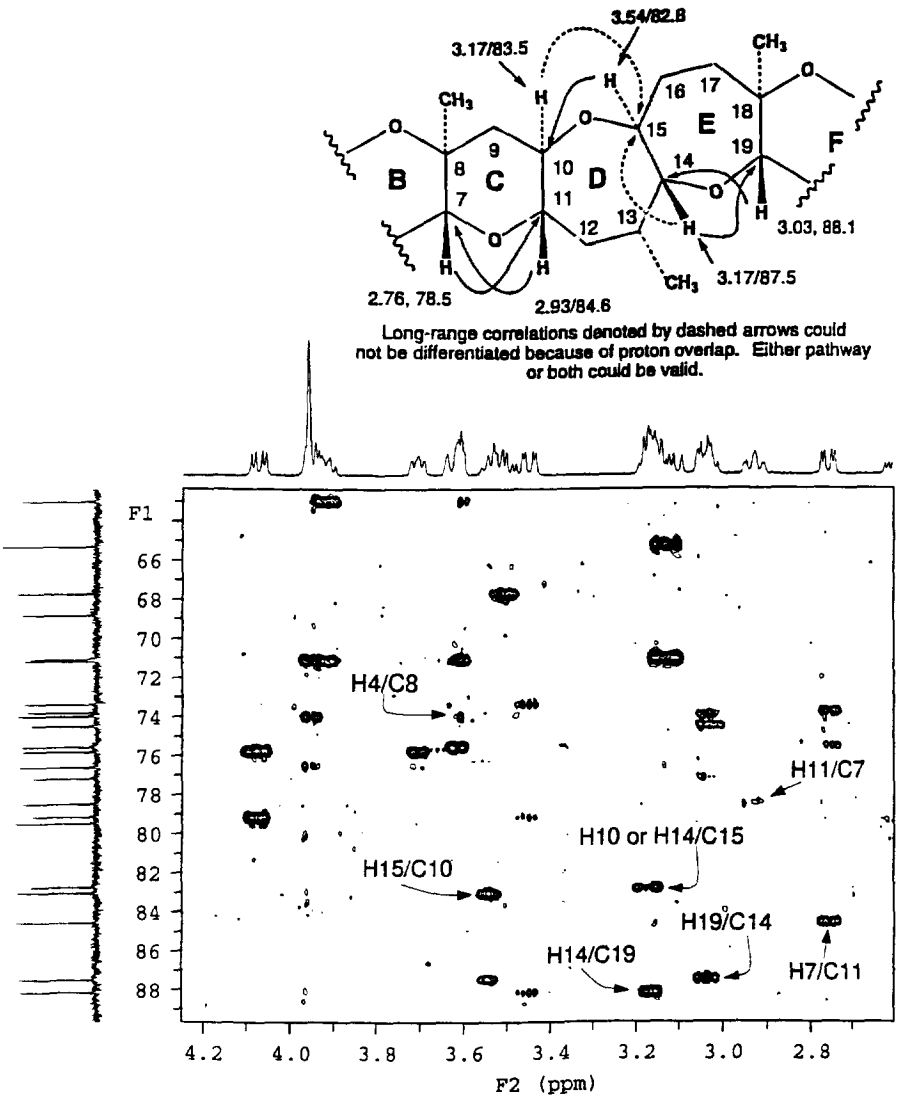

Figure 6. Excerpt from the HMBC spectrum described in Figure 2. Various correlations that are key to the assignment of ether linkages are detailed both on the spectrum and the structural fragment.

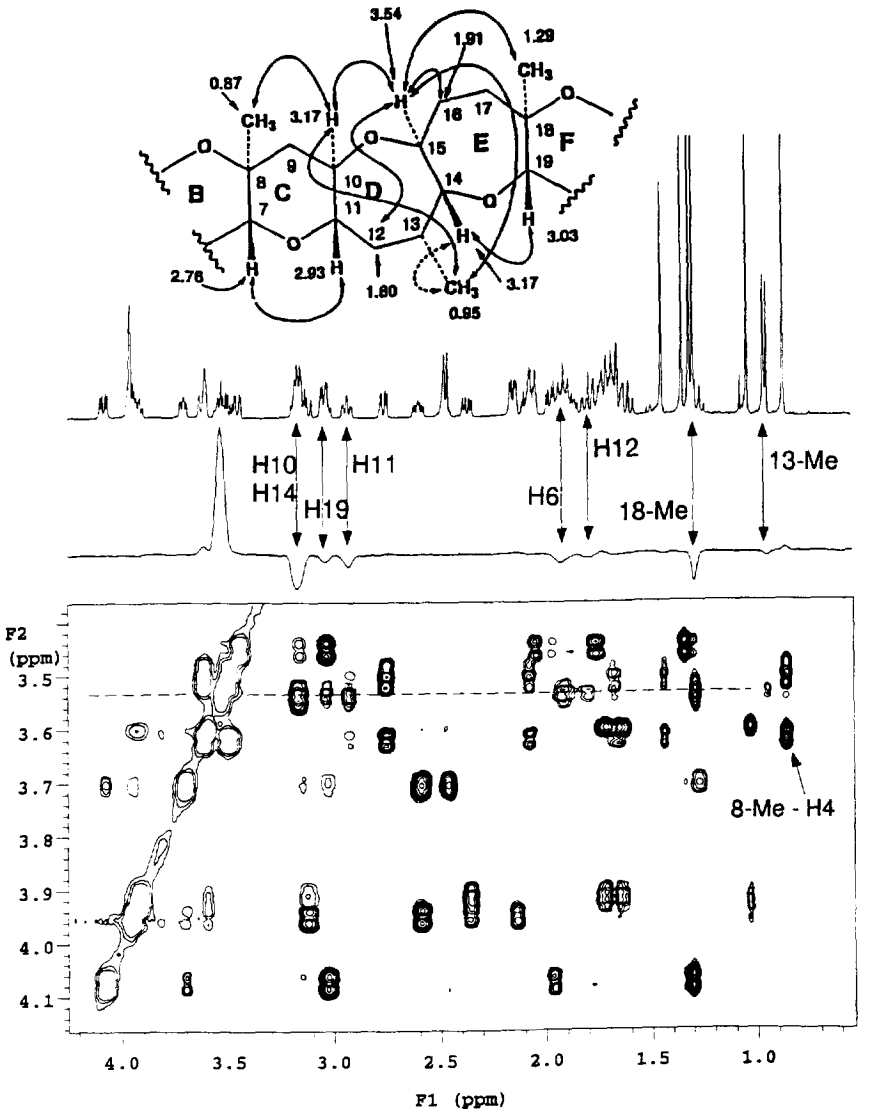

Figure 7. Expansion from a 2D ROESY spectrum acquired with 1. ROEs to H15 are detailed on the figure along with a structural fragment denoting these interactions with two-headed arrows. Spectral windows were 4012 Hz in both domains with acquisition times of 255 ms in F_2 and 86 ms in F_1. A 600 msec 1850 Hz spin-lock field was used. Thirty two transients were obtained on each of the 344 States-TPPI increments.

The first heteroatom bridges we must negotiate are those for the ester linkage between C1 and C5 in ring A and the ether bridge, C4-O-C8, in ring B. The establishment of the former is straightforward -- a long-range correlation in the HMBC spectrum establishes the correlation from H4 to the carbonyl, closing the δ-lactone and completing the assignment of ring A resonances. Continuing, the C4-O-C8 ether bridge is established through the long-range correlation from H4-C8 (see expansion of the oxygenated carbon region of the HMBC spectrum shown in Figure 6). Complementary confirmation of the C4-O-C8 ether bridge is provided from the H4-8-Me rOe extracted from the segment of the ROESY spectrum shown in Figure 7; the relative stereochemistry of H4 and the 8-Me substituents are inferred by the observed rOe between them.

Working further, the C7-O-C11 ether bridge is straightforward. Long-range correlations are observed in the HMBC spectrum from H7-C11 and H11-C7; likewise, a rOe was observed between H7 and H11 (not shown in Figure 7) resonating at 2.76 and 2.93, respectively. Again, the relative stereochemistry at the 7- and 11-positions is established from this rOe. Furthermore, the absence of an H7-8Me rOe suggests the opposite relative stereochemistry for H7 and H11 relative to the H4 and 8-methyl substituents.

Next, and more noteworthy is the C10-O-C15 ether bridge in the oxepin D ring. This linkage illustrates some of the sources of ambiguity which can plague structure elucidation with molecules as complex as brevetoxin-3 (**1**). While H15 is reasonably well resolved at 3.54 ppm (partially overlapped by H5 resonating at 3.51 ppm), both H10 and H14 are completely overlapped, resonating at 3.17 ppm. Hence, a potential three-bond heteronuclear coupling from H10-C15 could not be differentiated from a two-bond correlation from H14-C15. Likewise, a rOe between H15 and H10 could not be distinguished from one between H15 and H14, assuming that the stereochemistry allowed the latter interaction to occur. However, the C10-O-C15 ether bridge can be unequivocally established from a three-bond correlation ($^3J_{H15C10}$) from H15 to C10, the latter resonating at 83.02 ppm. The correlation pathway is also easily distinguished from the potential two-bond correlation from H15 to C14 ($^2J_{H15C14}$), C14 resonating at 87.49 ppm and well separated from C10. Finally, from a conventional ROESY spectrum, the relative stereochemistry of H10-H15 and H15-H14 remains open to question because of the overlap of H10 and H14. It would be possible, however, to define the rOe cross relaxation pathways and hence the relative stereochemistry by resorting to an HMQC-ROESY spectrum, if a sufficient or labeled sample were available.

The balance of the ether bridges may be established in a fashion analogous to one of those just described above with the exception of the C18-O-C22 ether bridge. In this case, both carbons in question are quaternary; the bridgehead methyl-bearing carbons preclude any usage of correlations from an HMBC experiment. Normally, in the absence of HMBC correlations, we would potentially have recourse to a rOe between the 18Me and 22Me from the ROESY spectrum. In this specific case, however, the methyls in question resonate at 1.29 and 1.30 ppm, respectively, precluding the use of a conventional ROESY spectrum to establish this bridge. We

could, if this were a total unknown and the correlation was necessary to complete the structure, resort to a ^{13}C-coupled full or F_1 region-selected HMQC-ROESY spectrum in a fashion analogous to that described for establishing coupling or nOe between overlapped resonances[23], or a rOe between identical protons in a C_2 symmetric molecule, as described in the work of Kawabata and co-workers.[22,24] In the case at hand, however, it was unnecessary to resort to any "exotic" NMR experiments. First, the structure is known; we were only concerned with a total assignment. Second, all of the other oxygen-bearing sites in the molecule were accounted for, leaving only the ether bridge between C18 and C22 to be established, which could be done by default.

A summary of the observed long-range heteronuclear couplings and rOe connectivities involving the ether/ester bridges of brevetoxin-3 (**1**) are shown on the structure presented in Figure 8. Long-range correlations are denoted in Figure 8 by unidirectional arrows from the proton to the carbon to which the proton is long-range coupled. ROe connectivities are denoted by double-headed arrows on the structure.

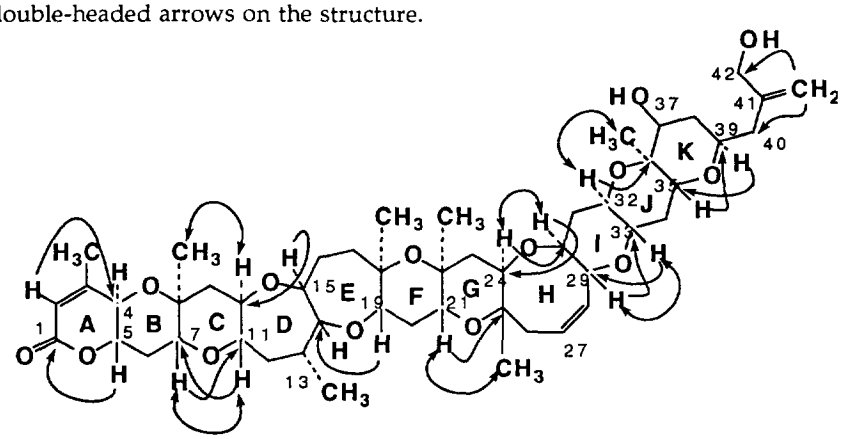

Figure 8. Summary long-range heteronuclear couplings (HMBC) and rOe responses used to establish the various ether bridges of brevetoxin-3 (**1**). Long-range heteronuclear couplings are denoted by unidirectional arrows; rOe pathways are denoted by double-headed arrows.

Using the approach just briefly described, it was possible to literally "walk around" the the brevetoxin-3 molecular skeleton generating a large partial structure in the process. Given the partial structure, the question of how the ether bridges were closed to elaborate the final constitution of the structure can be addressed from long-range heteronuclear correlations in the HMBC spectrum and/or cross-relaxation pathways in a NOESY/ROESY spectrum. It should also be noted that the data in hand provided considerable redundancy as it was generally possible to establish any bond by two, or more, different means. In practice we found that the homonuclear COSY spectrum provided a highly complementary means to check each vicinal connectivity. Thus, utilization of a PE COSY[25], which potentially provides resolved access to coupling constants in complex systems, would afford an independent method to probe relative stereochemistry. The method described, in conjunction with microprobe technology, offers a

powerful means of probing the structures of complex natural products, drug degradation products, metabolites, etc, especially when only severely limited quantities of material can be isolated.

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