Application of Two-Dimensional Nmr Spectroscopy in the Structural Determination of Marine Natural Products. Isolation and Total Structural Assignment of 4-Deoxyasbestinin Diterpenes from the Caribbean Gorgonian Briareum asbestinum

José J. Morales, Damaris Lorenzo, and Abimael D. Rodríguez

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APPLICATION OF TWO-DIMENSIONAL NMR SPECTROSCOPY IN THE STRUCTURAL DETERMINATION OF MARINE NATURAL PRODUCTS. ISOLATION AND TOTAL STRUCTURAL ASSIGNMENT OF 4-DEOXYASBESTININ DITERPENES FROM THE CARIBBEAN GORGONIAN *BRIAREUM ASBESTINUM*[1]

JOSÉ J. MORALES,[2] DAMARIS LORENZO,[3] and ABIMAEL D. RODRÍGUEZ*

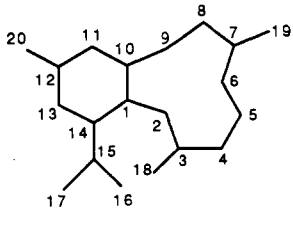
Department of Chemistry, University of Puerto Rico, Río Piedras, Puerto Rico 00931

ABSTRACT.—The structures of four novel asbestinin diterpenes isolated in the toxic extracts of the Caribbean gorgonian *Briareum asbestinum* are discussed. The major metabolite 11-acetoxy-4-deoxyasbestinin B [**2**] was subjected to a total structural assignment through the concerted application of several 2D nmr techniques that included ^{1}H-^{1}H COSY, RCT-COSY, RCT2 COSY, HC COSY (HMQC), NOESY, proton-detected long range heteronuclear chemical shift correlation (HMBC) and ^{13}C-^{13}C chemical shift correlation spectroscopy (INADEQUATE).

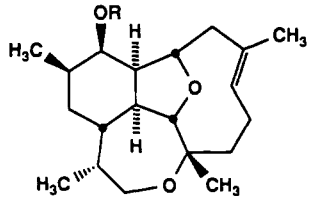
It is generally accepted that marine invertebrate organisms, having evolved over millions of years in the more stable environment provided by the sea, have been able to divert greater amounts of resources to the development of secondary metabolic pathways as part of their chemical survival strategy (1). Marine invertebrates, therefore, have spawned an impressive array of unique biological and chemical adaptations in response to their environment. A particularly prolific group of marine invertebrate organisms are the coelenterates, which already have yielded over 400 biosynthetic products previously unreported from terrestrial sources (2). Thus, the frequency of biological activity of coelenterate compounds may not be a mere coincidence and no doubt reflects a meticulous natural selection that is just now becoming apparent to us. This, in part, has stimulated our systematic chemical study of abundant toxic, soft-bodied invertebrates inhabiting the Caribbean waters near Puerto Rico.

In May 1990, during an underwater expedition near Puerto Rico, we collected the gorgonian *Briareum asbestinum* (Pallas) (phylum Cnidaria, class Anthozoa, subclass Alcyonaria, order Gorgonaceae), a common inhabitant of shallow Caribbean reefs. We found that at low concentrations (less than 25 μg/ml) the crude *Briareum* extract was highly toxic to CHO-K1 cells.

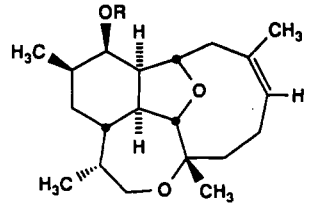
Previous chemical studies of the secondary metabolites of *B. asbestinum* had led to



Asbestinane skeleton



1 R=COC_3H_7
2 R=Ac



3 R=COC_3H_7
4 R=Ac

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[2]Graduate student sponsored by the NSF-EPSCoR Program of Puerto Rico.

[3]Undergraduate Research Associate.

the isolation of a series of bioactive chlorine-containing diterpenes of the briarein class (3–7), several novel steroids (8–10), taurobetaine (11), and a series of highly toxic non-chlorinated diterpenes having the uncommon asbestinane carbon skeleton (12,13). Generally, the structures of new asbestinin diterpenes have been established through a combination of degradative techniques and single-crystal X-ray diffraction methods. Selover *et al.* (13) have described the total assignment of the ^{1}H- and ^{13}C-nmr spectra of a series of asbestinin diterpenes; these efforts provide a partial data base which can be employed in the elucidation of new asbestinin analogues (13). A decade has passed since this report and there has, to the best of our knowledge, been no successful attempt at establishing an asbestinin structure using nmr spectroscopic techniques alone. On the other hand, with the advent of 2D nmr techniques in recent years, it has now become possible to establish the structures of these interesting compounds by spectroscopic means. We now report the complete structural assignments of the first 4-deoxyasbestinin diterpenes **1–4**, which have been accomplished exclusively on the basis of 2D ^{1}H-^{1}H, ^{1}H-^{13}C, and ^{13}C-^{13}C chemical shift correlation nmr spectroscopy.

At present only seven asbestinin diterpenes have been reported, all having been isolated from specimens of *B. asbestinum* collected near Belize (12) (Lighthouse Reef and Carrie Bow Cay) and Honduras (13) (Bay Islands). All these asbestinin diterpenes have in common an acetate or hydroxyl group at position C-4 and a butyrate ester group at C-11. Our structural work on the biotoxic extracts of *B. asbestinum* from Puerto Rico has revealed four new asbestinins that are characterized by the absence of an acetate (or hydroxyl) group at position C-4, and two of them, **2** and **4**, have the acetate group replacing the usual butyrate ester at C-11.

RESULTS AND DISCUSSION

A single collection of *B. asbestinum* from Palomino Key, Puerto Rico produced, after liophilization, 0.26 kg of the dry gorgonian coral. The MeOH/$CHCl_3$ solubles obtained upon extraction of the thawed animal yielded a crude extract which gave complex ^{1}H-nmr (300 MHz) and ^{13}C-nmr spectra (75 MHz) and significant in vitro cytotoxicity against CHO-K1 cells. Partitioning of an aqueous suspension of the MeOH/$CHCl_3$ extract against hexane and H_2O gave lipophilic solubles which accounted for 5.1% of the total organic content of *B. asbestinum*. The new 4-deoxyasbestinins were obtained pure after routine application of adsorption and reversed-phase chromatography. Their structures were determined from in-depth nmr spectral analyses following a strategy like that described earlier by us for diterpenes in the cembrane series (14). This provided all the ^{1}H- and ^{13}C-nmr chemical shift and coupling data shown in Tables 1 and 2.

Our initial structural assignment efforts began with the most abundant 11-acetoxy-4-deoxyasbestinin B [**2**] (0.43% dry wt). The molecular formula of this white crystalline solid, mp 150–152°, was determined by hrms analysis ($[M]^{+}$ *m/z* 362, $C_{22}H_{34}O_4$) and the ^{13}C-nmr data. The ir spectrum contained an ester band at 1730 cm^{-1}. The ^{13}C-nmr spectrum did not contain a signal for a tetrasubstituted carbon atom bearing carbon substituents, as found at the carbocyclic ring junction in the briarein series, nor did the mass spectrum indicate the presence of halogens. Therefore, asbestinin diterpene **2** could not be assigned a structure based on the briarein carbon skeleton.

ASSIGNMENT OF THE ^{13}C-NMR SPECTRUM.—The ^{13}C-nmr spectrum of **2** exhibited the expected 22 carbon resonances (Table 2) divided by APT (15) into three quaternary carbons (C-3, C-7, and C-21), nine methine carbons (C-1, C-2, C-6, C-9, C-10, C-11, C-12, C-14, and C-15), five methylenes (C-4, C-5, C-8, C-13, and C-16), and five methyl groups (C-17, C-18, C-19, C-20, and C-22). A closer inspection of the

TABLE 1. ^{1}H-nmr Spectral Data of the 4-Deoxyasbestinins **1–4**.[a]

Proton	Compound			
	1[b]	**2**[b]	**3**[c]	**4**[b]
H-1	2.16, ddd, 11.2, 11.2, 8.9, 1H	2.17, ddd, 10.7, 10.7, 8.5, 1H	2.34, m, 1H	2.34, ddd, 10.4, 10.4, 9.4, 1H
H-2	3.98, d, 8.6, 1H	3.99, d, 8.5, 1H	3.87, d, 8.6, 1H	3.87, d, 8.8, 1H
H-4α	2.04, m, 1H	2.05, m, 1H	1.76, m, 1H	1.75, t, 5.6, 1H
H-4β	1.49, m, 1H	1.50, m, 1H	1.76, m, 1H	1.75, t, 5.6, 1H
H-5α	2.35, m, 1H	2.39, m, 1H	1.95, m, 1H	1.94, br d, 6.7, 1H
H-5β	1.99, m, 1H	2.05, m, 1H	2.58, m, 1H	2.55, ddd, 14.3, 10.0, 4.9, 1H
H-6	5.33, m, 1H	5.35, dd, 9.1, 6.4, 1H	5.48, m, 1H	5.47, dd, 9.7, 6.7, 1H
H-8α	2.02, m, 1H	2.03, m, 1H	1.95, m, 1H	2.05, br d, 5.2, 1H
H-8β	2.43, dd, 13.2, 6.3, 1H	2.44, dd, 13.4, 5.9, 1H	2.52, m, 1H	2.50, br d, 15.2, 1H
H-9	4.03, dd, 6.0, 2.3, 1H	4.06, dd, 6.1, 2.4, 1H	4.08, ddd, 5.1, 2.5, 2.5, 1H	4.10, ddd, 5.6, 2.7, 2.7, 1H
H-10	2.04, m, 1H	2.05, m, 1H	2.0, m, 1H	1.97, m, 1H
H-11	5.33, dd, 5.0, 2.7, 1H	5.31, dd, 5.2, 2.7, 1H	5.32, dd, 4.8, 2.6, 1H	5.31, dd, 5.2, 2.7, 1H
H-12	2.02, m, 1H	2.02, m, 1H	2.05, m, 1H	2.04, m, 1H
H-13α	1.46, ddd, 13.6, 13.6, 9.6, 1H	1.47, ddd, 13.7, 13.7, 9.7, 1H	1.50, m, 1H	1.52, ddd, 13.4, 13.4, 9.7, 1H
H-13β	0.98, m, 1H	0.99, ddd, 13.4, 3.0, 1.5, 1H	0.99, m, 1H	1.01, ddd, 13.7, 3.4, 1.5, 1H
H-14	1.86, dddd, 1H	1.87, dddd, 1H	1.88, m, 1H	1.88, dddd, 1H
H-15	1.62, m, 1H	1.64, m, 1H	1.60, m, 1H	1.61, m, 1H
H-16α	3.48, dd, 13.6, 4.0, 1H	3.48, dd, 13.4, 3.7, 1H	3.48, dd, 13.1, 3.2, 1H	3.48, dd, 13.1, 3.4, 1H
H-16β	3.92, dd, 13.2, 1.3, 1H	3.93, dd, 13.4, 1.8, 1H	3.86, d, 14.1, 1H	3.86, d, 13.1, 1H
Me-17	0.86, d, 6.9, 3H	0.87, d, 7.0, 3H	0.91, d, 7.1, 3H	0.91, d, 7.0, 3H
Me-18	1.31, s, 3H	1.32, s, 3H	1.35, s, 3H	1.35, s, 3H
Me-19	1.74, s, 3H	1.75, s, 3H	1.76, s, 3H	1.75, s, 3H
Me-20	0.90, d, 7.3, 3H	0.92, d, 7.3, 3H	0.91, d, 7.1, 3H	0.92, d, 7.3, 3H
H-22	2.34, m, 2H	2.11, s, 3H	2.34, m, 2H	2.10, s, 3H
H-23	1.68, m, 2H	—	1.67, m, 2H	—
H-24	0.97, t, 7.3, 3H	—	0.98, t, 7.3, 3H	—

[a]Assignments were made on the basis of homonuclear chemical shift correlation methods [^{1}H-^{1}H COSY (J_3), RCT ^{1}H-^{1}H COSY (J_4) and RCT2 ^{1}H-^{1}H COSY (J_5)], heteronuclear chemical shift correlation methods (HMQC and HMBC), and NOESY experiments. The δ values are in ppm and are referenced to the residual $CHCl_3$ signal (7.26 ppm).

[b]500 MHz ^{1}H-nmr data.

[c]300 MHz ^{1}H-nmr data.

TABLE 2. ^{13}C-nmr Spectral Data of the 4-Deoxyasbestinins **1–4**.[a]

Carbon	Compound			
	1[b]	**2**[b]	**3**[c]	**4**[b]
C-1	38.64 (d)	38.71 (d)	40.67 (d)	40.56 (d)
C-2	93.79 (d)	94.01 (d)	92.16 (d)	92.25 (d)
C-3	79.54 (s)	79.54 (s)	76.85 (s)	76.43 (s)
C-4	38.64 (t)	38.58 (t)	38.71 (t)	38.54 (t)
C-5	23.14 (t)	23.14 (t)	23.28 (t)	23.37 (t)
C-6	132.67 (d)	132.67 (d)	130.97 (d)	130.81 (d)
C-7	124.87 (s)	124.76 (s)	128.74 (s)	128.70 (s)
C-8	44.65 (t)	44.71 (t)	37.46 (t)	37.51 (t)
C-9	80.83 (d)	80.88 (d)	81.11 (d)	81.01 (d)
C-10	48.28 (d)	48.41 (d)	45.76 (d)	45.79 (d)
C-11	73.48 (d)	73.88 (d)	73.28 (d)	73.56 (d)
C-12	31.13 (d)	31.19 (d)	31.46 (d)	31.37 (d)
C-13	31.71 (t)	31.74 (t)	31.63 (t)	31.57 (t)
C-14	38.03 (d)	38.01 (d)	38.12 (d)	38.01 (d)
C-15	37.20 (d)	37.24 (d)	37.46 (d)	37.35 (d)
C-16	67.84 (t)	67.78 (t)	67.95 (t)	67.93 (t)
C-17	11.05 (q)	11.03 (q)	10.95 (q)	10.96 (q)
C-18	25.24 (q)	25.20 (q)	26.17 (q)	26.07 (q)
C-19	18.74 (q)	18.66 (q)	29.03 (q)	28.89 (q)
C-20	17.99 (q)	17.89 (q)	18.06 (q)	17.91 (q)
C-21	174.01 (s)	171.33 (s)	173.31 (s)	171.33 (s)
C-22	36.66 (t)	21.20 (q)	36.72 (t)	21.32 (q)
C-23	18.47 (t)	—	18.50 (t)	—
C-24	13.77 (q)	—	13.75 (q)	—

[a]Assignments were made on the basis of heteronuclear chemical shift correlation methods, carbon atom multiplicities and chemical shift values. Multiplicities were obtained by an Attached Proton Test (APT) experiment. The δ values are in ppm and are referenced to the $CDCl_3$ signal (77.0 ppm).

[b]125 MHz ^{13}C-nmr data.

[c]75 MHz ^{13}C-nmr data.

^{13}C-nmr spectrum of **2** revealed one ester carbonyl carbon (δ 171.33), five oxygenated carbons (δ 94.01, 80.88, 79.54, 73.88, 67.78), and only two olefinic (δ 132.67, 124.76) carbons. Therefore, 4-deoxyasbestinin **2** contained two ether rings and two carbocyclic rings. The APT experiment indicated that one of the five oxygenated carbons (δ 79.54) is tertiary, three are secondary (δ 94.01, 80.88, 73.88), and the fourth (δ 67.78) belongs to a primary carbon. One of the olefinic carbons (δ 124.76) is a non-protonated vinyl and one is singly protonated (δ 132.67).

While many of the 22 carbon resonance assignments could be made unequivocally on the basis of ^{13}C chemical shift arguments, ^{13}C-APT, and conventional COSY spectra, most protonated carbon resonance assignments in 11-acetoxy-4-deoxyasbestinin B followed directly from the HC-COSY spectra. A proton-carbon chemical shift correlation via heteronuclear multiple quantum coherence (HMQC) experiment (16) correlated all proton resonances with their corresponding carbon resonances, including those in the aliphatic high field region (Figure 1). In all cases, it was possible to confirm independently all of the carbon resonance assignments from a series of proton-detected long-range heteronuclear chemical shift correlation spectra (HMBC) (17,18).

The most important correlations extracted from the wealth of information contained in the HMBC spectra were those which bridge quaternary carbons, heteroatoms, and functional groups that separate the isolated proton spin systems. Long-range correlations observed in the HMBC experiments performed are summarized in Table 3. Key substituents separating the aliphatic spin systems in **2** were the C-3 bridged quaternary

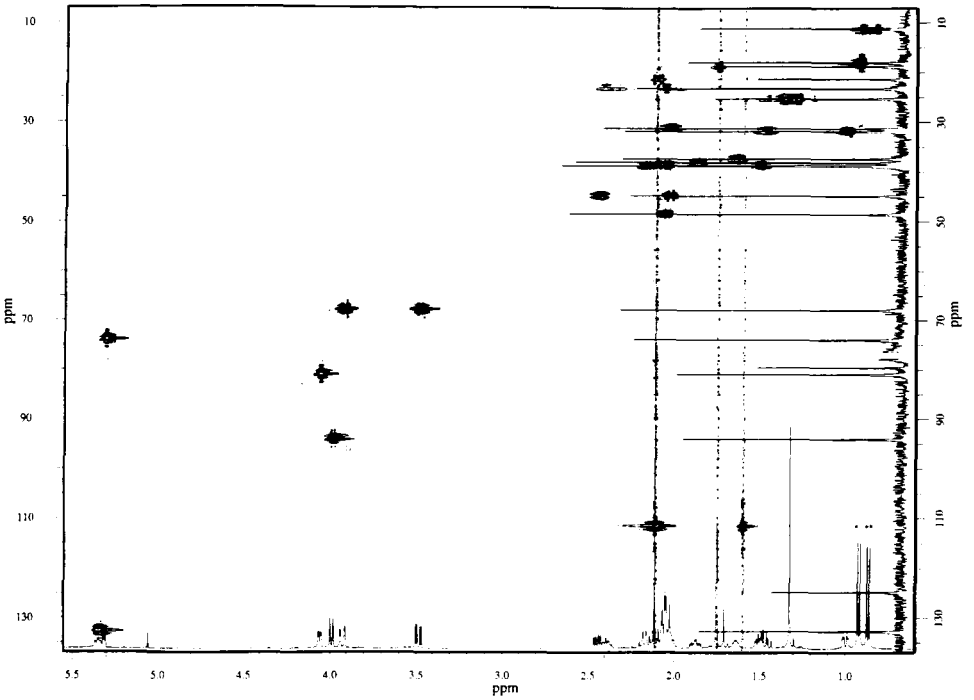

FIGURE 1. Proton-carbon heteronuclear correlation spectrum of 11-acetoxy-4-deoxyasbestinin B [**2**] acquired on a 30 mg sample using the proton-detected heteronuclear multiple quantum correlation experiment of Bax and Subramanian (16). The data was acquired at 500 MHz and the matrix consisted of 512 × 2K complex points.

carbon, the C-7 quaternary vinyl carbon, the O-ether linkage between C-2 and C-9, and the O-16 ether linkage. Linking structural fragments flanking the pivotal C-3 bridged quaternary carbon was particularly straightforward. Responses correlating with C-3 were the $^{2}J_{CH}$ couplings to the diastereotopic C-4 methylene protons, the C-2 methine proton, and the H-18 methyl protons. Extending our consideration to $^{3}J_{CH}$ couplings, we observed that C-3 also exhibits couplings to the diastereotopic H-16αβ protons in the HMBC spectrum. This established unequivocally the linking of C-3 with C-16 through an O-ether linkage in a fashion which is completely consistent with the presence of the proposed oxepane ring. In particular, further $^{2\text{-}3}J_{CH}$ couplings between C-2 and protons H-1, H-9 and H-14, and C-14 to the H-15 and H-16αβ protons, established unequivocally the presence of the seven-membered ether ring. On the other hand, the linking of the H-17 methyl group through C-15 of the oxepane ring was not particularly obvious from the HMBC spectra and was subsequently established by alternate 2D homonuclear chemical shift correlation methods.

Next it was necessary to establish the proposed partial structure of the homoallylic tetrahydrofuran ring. Correlations within the five-membered ether ring spin system: The C-2 was correlated with the H-1 and H-9 methine protons (the latter across the O-ether linkage), which in turn correlated with C-10. The C-9 carbon was also linked to the H-8αβ resonances which also long-range coupled to C-6 and C-7. Further, the H-6 vinyl proton was long-range coupled to C-8 in the HMBC spectrum. These correlations effectively established the structure of the homoallylic tetrahydrofuran skeletal moiety and position it within the molecular framework in a manner consistent with structure **2**.

The assembly of the 10-membered carbobicyclic ring in **2** by insertion of an ethylene bridge connected through C-3 and C-6 was also entirely straightforward from

TABLE 3. Protons to Which Long Range Correlations Were Observed in the HMBC Experiment, ^{1}H-^{1}H COSY, Long Range ^{1}H-^{1}H COSY, and Selected nOe Connectivities of 11-Acetoxy-4-Deoxyasbestinin B [**2**].

Atom	HMBC(^{1}H)[a]	COSY[b]	RCT-COSY[b]	RCT2-COSY[b]	nOe[c]
1	H-2,H-9,H-11,H-14	H-2,H-10,H-14	H-9,H-11,H-13α	H-4α,H-8α/β,H-12,H-17	H-10,H-17,H-19
2	H-1,H-4β,H-9,H-14,H-18	H-1	H-4α,H-10,H-14	H-5β,H-9	H-9,H-14,H-16β,H-18
3	H-1,H-2,H-4α/β,H-5α/β,H-16α/β,H-18	—	—	—	—
4α	H-2,H-5α/β	H-4β,H-5α/β	H-2,H-6,H-18	H-1	—
4β	—	H-4α,H-5α/β	—	—	H-18
5α	H-4α/β,H-6	H-4α/β,H-5β,H-6	—	H-19	H-19
5β	—	H-4α/β,H-5α,H-6	—	H-2,H-18,H-19	H-6,H-18
6	H-4β,H-5α/β,H-8α/β,H-19	H-5α/β,H-19	H-4α,H-8α/β,H-19	—	H-5β
7	H-5α/β,H-8α/β,H-9,H-19	—	—	—	—
8α	H-6,H-19	H-8β,H-9	H-6,H-19	H-1,H-11	H-9 (weak)
8β	—	H-8α,H-9	H-6	H-1	H-9 (strong)
9	H-8α/β,H-10,H-11	H-8α/β,H-10	H-1	H-2,H-12,H-14	H-2,H-8α/β
10	H-1,H-2,H-8α/β,H-9,H-11	H-1,H-9,H-11	H-2,H-14	H-13α/β	H-1,H-12
11	H-1,H-9,H-20	H-10,H-12	H-1,H-13α,H-20	H-8α,H-14	—
12	H-13α	H-11,H-13α,H-20	H-14	H-1,H-9	H-10,H-13α/β
13α	H-11,H-14	H-12,H-13β,H-14	H-1,H-11,H-20	H-10	H-12,H-17
13β	—	H-13α,H-14	—	H-10	H-12
14	H-1,H-2,H-15,H-16α/β	H-1,H-13α/β,H-15	H-2,H-10,H-12,H-16α/β,H-17	H-9,H-11,H-20	H-2,H-16β
15	H-14	H-14,H-16α/β,H-17	—	—	H-16α/β
16α	H-17	H-15,H-16β	H-14,H-17	—	H-15,H-16β,H-17,H-18
16β	—	H-15,H-16α	H-17	—	H-2,H-14,H-15,H-16α,H-18
17	—	H-15	H-14,H-16α/β	H-1	H-1,H-13α,H-16α
18	H-2	—	H-4α	H-5β	H-2,H-4β,H-5β,H-16α/β
19	H-6,H-8α/β	H-6	H-6,H-8α	H-5α/β	H-1,H-5α
20	H-13α/β	H-12	H-11,H-13α	H-14	—
21	H-11,H-22	—	—	—	—
22	—	—	—	—	—

[a]The experiment was optimized for long-range couplings of ~7 Hz with a fixed delay $\Delta = 67.9$ msec. The low pass J-filter in the experiment to eliminate responses from direct ($^1J_{CH}$) pairs was optimized for 142 Hz (3.50 msec.)

[b]COSY spectra measured in $CDCl_3$ at 500-MHz.

[c]Obtained from the 2D nOe spectrum for a sample of ca. 30 mg of **2** in 0.5 ml of $CDCl_3$ using a mixing time of 500 msec; a 5-mm probe was used.

the long-range HC-COSY data shown in Table 3. Finally, the H-19 methyl group is linked to the 10-membered carbobicyclic ring through the C-7 vinyl carbon by long-range couplings to carbons C-6, C-7, and C-8.

As depicted in **2**, the structure of 11-acetoxy-4-deoxyasbestinin B is a complicated tetracyclic array. Having demonstrated spectroscopically how to assemble three of the four rings in **2** through the use of ^{1}H-^{13}C chemical shift correlation experiments only, all that remained to be assigned was the location and size of the remaining carbocyclic system. To this end, we observed that the only response correlated with C-10 was the $^{2}J_{CH}$ couplings to the C-11 methine proton, which in turn showed three-bond ^{1}H-^{13}C coupling to C-13. A two-bond ^{1}H-^{13}C coupling between one of the methylene protons at C-13 (H-13α) and C-12 established only partially the structure of the six-membered ring. However, the 7 Hz optimized HMBC spectrum was not particularly informative with regard to establishing the C-11–C-12 and C-13–C-14 carbon connectivities unequivocally within the cyclohexane skeletal moiety. The linking of the C-20 methyl group to the cyclohexane ring as shown could not be established unambiguously from the HMBC spectrum either.

Recognizing that the extraction of the carbon-carbon connectivities encompassing carbons C-10 through C-14 was complicated by the general absence of long-range ^{1}H-^{13}C coupling within the cyclohexane skeletal moiety, we turned our attention to alternative 2D homonuclear chemical shift correlation methods. An alternate means of establishing the carbon connectivities across C-10–C-14 while confirming simultaneously the entire carbon connectivity network already established from the HMBC experiments would be to arrive at the carbon-carbon connectivity directly from ^{13}C-^{13}C couplings. Quite obviously, since one of the problems associated with performing ^{13}C-^{13}C double quantum coherence nmr experiments is the inherently low sensitivity regardless of which variant of the experiment is utilized, only the INADEQUATE spectrum (19–21) of the most abundant of the 4-deoxyasbestinin diterpenes, **2**, could be recorded. Fortunately, 11-acetoxy-4-deoxyasbestinin B, as a model compound, showed very generous solubility in $CDCl_3$ and quite favorable relaxation characteristics, since we were dealing almost exclusively with protonated carbon resonances.

The INADEQUATE spectrum of **2** showed the expected cross peaks of 21 pairs of carbons as illustrated in Figure 2. The duration of the fixed delay, τ, was optimized as a function of ¼ (J_{CC}). Because the typical carbon-carbon coupling constants in **2** should range from about 40 Hz for a pair of coupled aliphatic carbons (there are a total of 17 such pairs in **2**) to about 70 Hz for coupled vinylic carbons (only one pair), τ was selected for the average aliphatic C-C coupling (45 Hz). Therefore, the correlation across Δ^{6} was of low intensity and is not visible in the plot (Figure 3). Correlated long-range ^{1}H-^{13}C spectra further elucidated the connection between C-6 and C-7 (Table 3). The INADEQUATE spectrum afforded readily the unequivocal assignments of all carbon resonances in **2** and established unambiguously the balance of the protonated carbons (C-10 through C-14 including the C-15 and C-20 methyl groups) of the tetracyclic array. Connection of each carbon pair clarified the sequence of carbon atoms and permitted the assignment of all carbon atoms in 11-acetoxy-4-deoxyasbestinin B unambiguously.

ASSIGNMENT OF THE ^{1}H-NMR SPECTRUM.—In order to achieve the complete assignments of the proton spectrum of **2**, we employed a combination of 2D proton experiments which included conventional COSY, RCT, and RCT2-COSY (22–24). These techniques alone allowed us to unravel all the connectivity pathways in the highly congested high-field spectral region (Table 3). Excluding the three methyl group resonances absorbing as singlets (C-18, C-19, and C-22), the aliphatic portion of

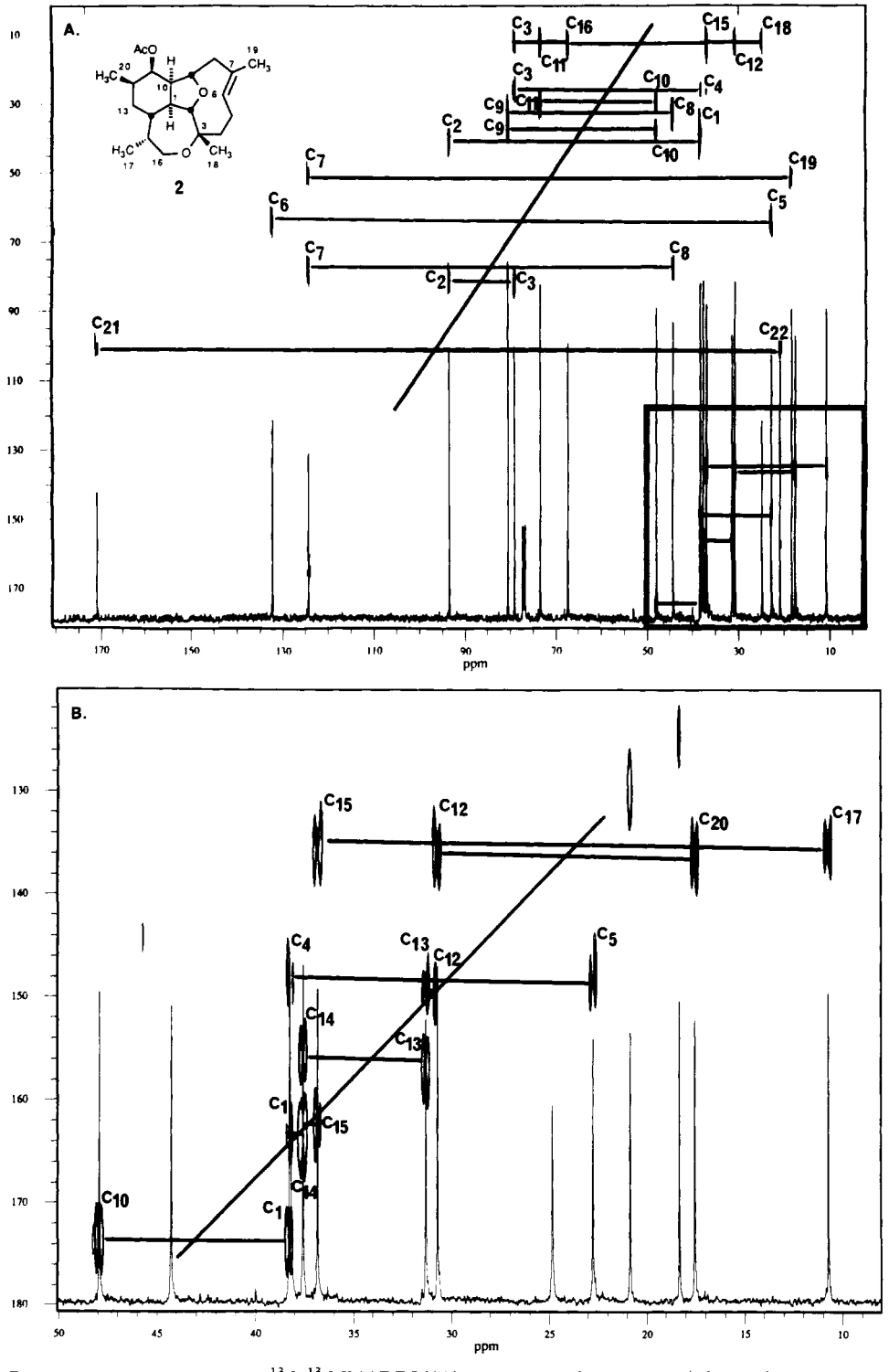

FIGURE 2. (A) 125.25 MHz ^{13}C-^{13}C INADEQUATE spectrum of 11-acetoxy-4-deoxyasbestinin B [**2**] acquired on a 200 mg sample and recorded in $CDCl_3$. The data was taken as 256 × 4K complex points and processed to afford a final matrix consisting of 512 × 4K points. Total acquisition time was ca. 8 h. (B) Expansion of the ^{13}C-^{13}C INADEQUATE spectrum of **2** in the congested region from 50 to 10 ppm.

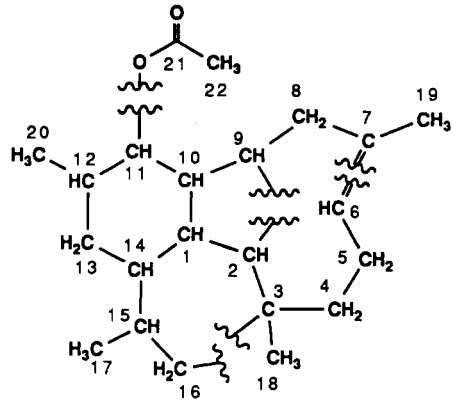

FIGURE 3. Connectivity networks identifying the asbestinane carbon skeleton in 11-acetoxy-4-deoxyasbestinin B [**2**]. The 2D-INADEQUATE spectrum of **2** showed the cross peaks of 21 pairs of carbons. Since τ was selected for the average aliphatic C-C coupling (ca. 45 Hz), the correlation across the double bond was of low intensity and is not visible in the plot.

the ^{1}H-nmr spectrum of **2** is subdivisible into two essentially isolated spin systems at 500 MHz. Constituents of these spin systems are readily established using the H-2 methine proton resonating at 3.99 and the H-6 vinyl proton resonating at 5.35 as starting points in the analysis of the COSY spectrum of **2**. Beginning from the pivotal H-2 angular proton resonating at 3.99, we first established a correlation to the H-1 methine proton, which resonates at 2.17. The H-1 methine proton was correlated, in turn, to a methine proton resonating at 2.05, which we assigned to the C-10 methine proton, and to a further methine proton resonating at 1.87, which may be assigned as H-14. At this point, establishing the proton-proton connectivity network around the tricyclic array constituted by the fused ether rings and the cyclohexane skeletal moiety, was straightforward. Linking structural fragments flanking the C-14 methine proton resulted from strong correlations observed in the COSY spectrum to the geminal methylene pair resonating at 1.47 and 0.99, which we assigned to the C-13 methylene protons, and to a further methine proton resonating at 1.64, which we assigned as H-15. Finally, the H-15 methine resonance was correlated with the C-17 methyl protons, which resonate at 0.87, and to a geminal methylene pair resonating at 3.93 and 3.48, which we assigned to the C-16 methylene protons. Since the correlations to these methylene protons stopped here, the C-16 resonances must mark a break in the proton-proton connectivity network. These correlations, combined with the long-range ^{1}H-^{13}C coupling detected between C-3 and the H-16$\alpha\beta$ protons confirmed that the C-3 quaternary carbon is bridged to the C-16 carbon through an ether linkage, thereby linking together the two structural fragments which make up the oxepane ring.

The C-13 methylene proton resonating at 1.47 was readily correlated with a complex multiplet resonating at 2.02, which may be assigned as the C-12 methine proton. The H-12 methine proton was correlated, in turn, to a methyl doublet resonating at 0.92 ascribable to the Me-20 group, and a doublet of doublets resonating at 5.31 assigned as the H-11 methine proton. At this point it should be noted that since the H-11 proton shows $^1J_{CH}$ coupling to a carbon doublet resonating at δ 73.88 in the HMQC

experiment, these correlations combined establish unequivocally the position of the acetoxy group at C-11 in a fashion which is completely consistent with structure **2**. Finally, the H-11 methine proton produced strong responses that correlated with the H-10 methine proton. This correlation information from the COSY spectrum effectively established the structure of the cyclohexane skeletal moiety and positioned it within the molecular framework shown in structure **2**. Unlike the various heteronuclear chemical shift correlation experiments (HC-COSY) described previously, these efforts established unequivocally the structure of the six-membered ring and assigned the protons (and carbons) of the C-11–C-13 propylene bridge.

The rest of the constituents of the largest spin system in **2** were readily established from the analysis of its COSY spectrum. Direct proton-proton connectivity linking H-10 with the methine proton H-9 resonating at 4.06 was clearly observed from the COSY spectrum. The H-9 resonance was, in turn, coupled to the H-8 geminal methylene resonances located at δ 2.44 and 2.03. In addition to the strong vicinal couplings described, the H-8 methylene proton resonating at 2.03 also showed long-range spin coupling to the more remote vinyl methyl protons at C-19 as detected from the RCT-COSY spectrum of **2**. Further long-range $^4J_{HH}$ couplings consistent with the presence of a tetrahydrofuran skeletal moiety were also detected between methine protons H-1 and H-9 and H-2 and H-10 in the RCT-COSY spectrum (Table 3). Since the H-8–H-9 coupling response leads to a termination point, C-7 must be a quaternary carbon.

The constituents of the remaining and much simpler isolated spin system in 4-deoxyasbestinin **2** were readily established using the vinyl proton resonating at 5.35 as starting point in the analysis of the COSY spectrum. The H-6 vinyl proton was readily correlated with a geminal pair of protons resonating at 2.39 and 2.05, which were ascribable to the diastereotopic C-5 methylene protons. The latter pair was correlated, in turn, to another geminal methylene pair resonating at 2.05 and 1.50, which we assigned to the C-4 methylene protons. Since both C-4 and C-6 mark break points in the proton-proton connectivity network, at this point we bridged these sites into the remaining molecular framework. Two means of establishing the balance of the connectivity network were available. First, the 7 Hz optimized HMBC spectrum afforded correlations between H-6 and carbons C-8 and C-19, thus directly establishing the correlation pathway required. Moreover, long-range $^3J_{CH}$ coupling between H-2 and carbon C-4 agreed completely with the assembly of the two isolated spin systems in a manner consistent with structure **2**. Finally, the assembly of the structural fragments as shown was consistent with the ^{1}H-^{1}H long-range coupling observed between H-6 and the C-19 methyl protons detected in the conventional COSY and RCT-COSY spectra of **2**.

STEREOCHEMICAL ASSIGNMENTS.—At this point, the ^{1}H-nmr spectrum of 4-deoxyasbestinin **2** had been unequivocally assigned and the structure independently generated and shown to be consistent with the one proposed on the basis of 2D ^{1}H-^{13}C and ^{13}C-^{13}C chemical shift correlation spectroscopy. All of the proton identities had been established, but the stereochemical orientations of the H-1, H-2, H-9, H-10, H-11, H-12, H-14, and H-15 protons remained to be assigned, as did those of the five geminal methylene pairings. Thereafter, the major structural features that remained to be determined were the stereochemical orientation of the C-3-methyl substituent and the geometry of the Δ^6 trisubstituted double bond. Such information could presumably be obtained from a one-dimensional nuclear Overhauser difference (nOeds) spectrum (25). However, since there have been a relative few successful applications of 2D nOe data for stereochemical problems with small to medium-sized organic molecules (26), it seemed more useful to gather most of the stereochemical assignments needed via a NOESY experiment (27,28). In general, strong nOe responses were observed in the

NOESY spectrum of **2** using a mixing time of 500 msec which correlated the desired resonances (Table 3). These responses resolved the questions regarding the stereochemical orientations of most of the key protons in **2**.

A convenient starting point from which the relative stereochemical assignments can be made is the proton at position 1 which we have assigned as α (13). Given the orientation of the 1α proton as a starting point, we could immediately establish the orientations of the key protons at the 2 and 14 positions as β because they failed to exhibit an nOe response correlating them with 1α. However, an intense nOe response was observed between H-2 and the H-14 resonance at 1.87, which confirmed the orientation of the latter resonances in the β orientation. In agreement with the stereochemistry around the oxepane ring shown in **2**, an nOe response was also observed between the downfield H-16 proton and both the H-2 and H-14 protons; hence it was assigned as H-16β. Further confirmation of this assignment was provided by a strong nOe linking the 3-Me substituent and H-16β. The determination of the α orientation of the 15-Me substituent in the oxepane ring was also consistent with the proposed stereochemistry on the basis of observed nOe responses with the H-13α proton resonating at 1.47, H-1, and H-16α.

An interesting structural feature established by the NOESY data was the boat conformation adopted by the cyclohexane structural unit in **2**. In general, nOe responses between the H-1/H-10, H-10/H-12, H-12/H-13α, and H-1/H-13α pairs, confirming the orientation of the latter resonances in the α stereochemistry, could not be established unambiguously due to the close proximity of the H-1, H-10, and H-12 resonances. On the other hand, the absence of an nOe response between the Me-20 protons at δ 0.92 and the H-9 and H-14 resonances at δ 4.06 and 1.87, respectively, established that the cyclohexane structural unit in **2** does not adopt a chair-like conformation.

Further useful correlations established the stereochemical assignments of additional protons in 4-deoxyasbestinin **2**. Given the orientation of the 14β proton, we could establish the orientation of the key proton at the 11 position as α because it failed to exhibit an nOe response correlating it with 14β, thus establishing the orientation of the 11-acetoxy substituent in a flagpole position. Given this key position, we next embarked upon the stereochemical assignment of the remaining proton resonance H-9 of the tetrahydrofuran structural unit. Because H-9, resonating at 4.06, failed to exhibit an nOe response correlating it with H-10α and instead produced a strong nOe response which correlated it with H-2β, we established the orientation of this key resonance in the β orientation.

At this point, the relative stereochemical orientations of all the key protons in 4-deoxyasbestinin **2** have been established except for those located in the six-carbon chain between C-3 and C-8 as well as the two methyl substituents attached to it (Me-18 and Me-19). Intense nOe responses were observed between the upfield H-5 proton and the 3-Me substituent which, in turn, exhibited a similarly strong nOe response to H-2β. This established the orientation of the latter resonances in the β orientation. Hence, H-5β was assigned as the proton resonating at 2.05 while H-5α, which showed an nOe response to the Me-19 protons, was assigned to the resonance at 2.39. Unfortunately, there were no intense nOe responses in the spectrum recorded that gave any information about the stereochemical identity of the H-4αβ protons. Also, because H-5β and the H-4 resonating downfield at δ 2.05 were accidentally isochronous, combined with the fact that about equally strong coupling responses between H-4β and the H-5αβ protons can be expected upon examination of a Dreiding model, the ^{1}H-^{1}H COSY spectrum also failed to give information about the stereochemical orientation of the H-4αβ protons. However, a very weak nOe response was observed between the 3-Me sub-

stituent and the H-4 resonance at δ 1.50, which establishes the orientation of the latter resonance in the β orientation. Hence, H-4α was assigned as the proton resonating at 2.05 while H-4β was assigned to the response at 1.50. The relative stereochemical orientations of the H-8αβ protons were established similarly on the basis of a strong nOe response between the downfield H-8 and the H-9 protons which, in turn, exhibited a much weaker nOe response to the upfield H-8 proton. Moreover, in the COSY spectrum, a strong coupling response was observed between H-9 and the H-8 resonating downfield at 2.44. In a Dreiding model of **2**, the dihedral angle between H-9 and H-8β is about 30°, which would account for a large coupling. In contrast, the dihedral angle between H-9 and H-8α is essentially 90°, and only a weak coupling response was observed in the COSY spectrum. Therefore, H-8β was assigned as the proton resonating at 2.44 while H-8α was assigned to the resonance at 2.03. Finally, the one other structural feature confirmed by the NOESY data was the orientation of the methyl group of the Δ^6 trisubstituted double bond. A lack of an nOe response between the H-6 proton and the Me-19 group confirmed the *E* orientation of the double bond. The geometry of the trisubstituted double bond in 4-deoxyasbestinin **2** was also assigned trans from its signals in the ^{13}C-nmr spectrum which showed significant shielding of the Me-19 group caused by vicinal carbons in the same way as in *trans*-polyisoprene (29). A perspective drawing of 11-acetoxy-4-deoxyasbestinin B [**2**] showing the stereochemical orientations of the protons as deduced from the nOe and COSY studies just described is presented in Figure 4.

The structure of 11-acetoxy-4-deoxyasbestinin D [**4**] (0.072% dry wt) showed it to be an isomer of 11-acetoxy-4-deoxyasbestinin B [**2**]. The ^{1}H-nmr spectrum of **4** contained signals for an acetate ester [δ 2.10 (s, 3H)], and the ir spectrum contained an ester band at 1738 cm^{-1}. Comparison of the ^{1}H-nmr spectra of 11-acetoxy-4-deoxyasbestinins **2** and **4** revealed that the major differences were associated with the carbocyclic 10-membered ring. In the spectrum of 11-acetoxy-4-deoxyasbestinin B the α-acetoxy and olefinic protons nearly overlapped at δ 5.31, whereas the spectrum of 11-acetoxy-4-deoxyasbestinin D contained an olefinic signal at δ 5.47 (dd, 1H, J = 9.7 and 6.7 Hz) and an unchanged α-acetoxy signal at δ 5.31 (dd, 1H, J = 5.2 and 2.7

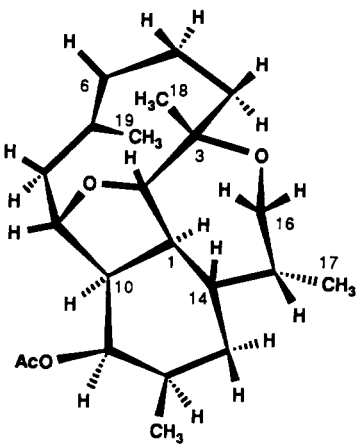

FIGURE 4. A perspective drawing of 11-acetoxy-4-deoxyasbestinin B [**2**] showing the stereochemical orientation of the protons as deduced from the NOESY and COSY studies described.

Hz). The presence of two allylic proton signals at 2.55 (ddd, 1H, $J = 14.3$, 10.0, and 4.9 Hz) and 1.94 (br d, 1H, $J = 6.7$ Hz) coupled to the olefinic proton signal suggested that 11-acetoxy-4-deoxyasbestinin D [**4**] contained a Δ^6 olefinic bond and must therefore be a geometrical isomer of 11-acetoxy-4-deoxyasbestinin B [**2**]. The *E* and *Z* geometry of the double bonds of **2** and **4**, respectively, was clearly shown by the ^{13}C-nmr chemical shift difference of the Me-19 groups (δ 18.66 vs. 28.89) and the signal at δ 44.71 associated to the C-8 methylene in **2** which appears shifted upfield to 37.51 in **4** (29). The near identity of the other methyl shifts in **2** vs. **4** at Me-17 (δ 11.03 vs. 10.96), Me-18 (δ 25.20 vs. 26.07), and Me-20 (δ 17.89 vs. 17.91) is in line with the identical stereochemistry of these groups. The total structural assignment of 11-acetoxy-4-deoxyasbestinin D [**4**] was achieved through the concerted application of 2D-nmr techniques that included conventional COSY, RCT-COSY, RCT2-COSY, HC-COSY, NOESY, and HMBC. The unequivocal assignments of the ^{1}H- and ^{13}C-nmr spectra of **4** are reported in Tables 1 and 2.

Minor metabolite 4-deoxyasbestinin A [**1**] (0.034% dry wt) had the molecular formula $C_{24}H_{38}O_4$. The ^{1}H-nmr spectrum of **1** was almost identical with that of 11-acetoxy-4-deoxyasbestinin B [**2**], with the exception that it did not contain an acetate signal. Instead, it contained signals for a butyrate ester [δ 0.97 (t, 3H, $J = 7.3$ Hz), 1.68 (m, 2H), and 2.34 (m, 2H)]. The ir spectrum contained an ester band at 1731 cm^{-1}. In the COSY spectrum of 4-deoxyasbestinin A [**1**], the α-butyroxy proton (overlapped with the olefinic proton at δ 5.33) produced strong responses that correlated with the H-10 methine proton (δ 2.04). Moreover, the H-11 α-butyroxy proton shows $^1J_{CH}$ coupling to a carbon doublet resonating at δ 73.48 in the HMQC experiment, ascribable to the C-11 secondary carbon. These correlations combined established unequivocally the position of the butyroxy group at C-11 in a fashion which is consistent with structure **1**. That the stereochemistry for **1** at the chiral centers 1, 2, 9, 10, 11, and 14 was identical to that of 11-acetoxy-4-deoxyasbestinin B [**2**] could be shown by the similarity of the *J* values involving protons at these sites. The stereochemistry at Me-17 (δ 11.05), Me-18 (δ 25.24), and Me-20 (δ 17.99) was unchanged. The *E* geometry of the double bond of **1** is clearly shown by the ^{13}C-nmr chemical shift of the Me-19 (δ 18.74) and the C-8 methylene carbon (δ 44.65). The rigorous assignments of the ^{1}H- and ^{13}C-nmr spectra of 4-deoxyasbestinin A (Tables 1 and 2) were established by application of most of the 2D nmr techniques described earlier.

The least abundant of the secondary metabolites isolated from *B. asbestinum*, 4-deoxyasbestinin C [**3**] (0.003% dry wt), was also shown to be an isomer of 4-deoxyasbestinin A [**1**]. Again, comparison of the ^{1}H-nmr spectra of 4-deoxyasbestinin C [**3**] and 4-deoxyasbestinin A [**1**] revealed that the major differences were associated with the carbocyclic 10-membered ring. The ^{1}H-nmr spectrum of **3**, which did not contain an acetate signal, was almost identical with that of 4-deoxyasbestinin A [**1**], with the exception that one of the overlapping signals at δ 5.33 in the spectrum of **1** now occurred at δ 5.48 (m, 1H). On the other hand, the ^{1}H-nmr spectrum of **3** contained signals for a butyrate ester [δ 0.98 (t, 3H, $J = 7.3$ Hz), 1.67 (m, 2H), and 2.34 (m, 2H)]. The ir spectrum contained an ester band at 1730 cm^{-1}. The presence of an allylic proton signal at δ 2.34 (m, 2H) coupled to the olefinic proton signal suggested that 4-deoxyasbestinin C [**3**] contained a Δ^6 olefinic bond also and must therefore be a geometrical isomer of 4-deoxyasbestinin A [**1**]. The ^{1}H- and ^{13}C-nmr data for **3** showed that all common stereochemistry elements, excepting that of the geometry of the double bond, were also unchanged. The *Z* stereochemistry of the latter was established by the chemical shift difference of the Me-19 (δ 29.03 vs. 18.74) and of the C-8 methylene carbon (δ 37.46 vs. 44.65).

In the present work we describe the results of a concerted 2D nmr study on four new

structurally interesting asbestinin diterpenes. Provided in the process are the unequivocal total assignment of the ^{1}H- and ^{13}C-nmr spectra of each molecule which have been accomplished exclusively on the basis of 2D ^{1}H-^{1}H, ^{1}H-^{13}C, and ^{13}C-^{13}C chemical shift correlation nmr spectroscopy. Where proton resonance assignments could be unequivocally made, protonated carbon resonance assignments followed directly from the HC-COSY spectra. In all cases, it was possible to confirm independently all of the carbon resonance assignments from the HMBC, and in the case of **2** from ^{13}C-^{13}C chemical shift correlation spectroscopy (INADEQUATE). The stereochemical orientation of the 31 protons directly connected to the complex tetracyclic array in each molecule was also established from the COSY and NOESY experiments described. These studies will help to establish a data base of sufficent size to be useful in future efforts directed at the ^{1}H- and ^{13}C-nmr-based structure elucidaton of new asbestinins of unknown structure.

All of the above 4-deoxyasbestinin derivatives showed pharmacological activity. Compounds **1**, **2**, **3**, and **4** show significant in vitro cytotoxicity against CHO-K1 cells (ED_{50} 3.35, 2.50, 3.55, and 4.82 μg/ml, respectively) and strong antimicrobial activity against *Klebsiella pneumoniae*. On the other hand, compound **2** proved inactive in the National Cancer Institute's test for agents active against the human immunodeficiency virus (HIV).

EXPERIMENTAL

GENERAL PROCEDURES AND ISOLATION.—^{1}H-nmr spectra were recorded at 500 MHz and ^{13}C spectra at 125 MHz on a General Electric GN OMEGA 500 spectrometer; chemical shifts are reported in ppm (δ) for $CDCl_3$ solutions downfield from internal TMS. Ir spectra were measured on a Nicolet 600 FT-IR spectrometer. High resolution mass measurements were supplied by Dr. Ronald L. Cerny from the Midwest Center for Mass Spectrometry, Department of Chemistry, University of Nebraska–Lincoln. Size exclusion chromatography was performed on Bio Rad Bio-Beads S-X2 (200–400 mesh), cc on Analtech Si gel (35-75 mesh), and tlc analyses using Analtech glass packed precoated Si gel plates. All hplc was performed on a Whatman Magnum 9 10/50 Partisil semipreparative column (10 mm × 25 cm) and a Beckman Ultrasphere-ODS semipreparative column (10 mm × 50 cm). All solvents used were either spectral grade or were distilled from glass prior to use.

COLLECTION AND EXTRACTION OF *B. ASBESTINUM*.—The Caribbean gorgonian *B. asbestinum* was collected by hand using SCUBA at depths of 3–5 m in May 1990 from Palomino Key, Puerto Rico. A voucher specimen is stored at the Chemistry Department of the University of Puerto Rico. The gorgonian was kept frozen until extraction. The freeze-dried animal (263.0 g) was blended with MeOH-$CHCl_3$ (1:1) (3 × 1 liter), and after filtration the crude extract was evaporated under vacuum to yield a residue (26.2 g) that was partitioned between hexane and H_2O. The hexane extract was subsequently filtered, and the filtrate was concentrated in vacuo to yield 13.5 g of a dark green oily residue. Almost half of the residue (5.32 g) was dissolved in a small volume of toluene and the resulting concentrate was fractionated by size exclusion chromatography on a Bio-Beads SX-2 column with toluene as eluent. The combined diterpene-containing fractions (tlc guided) were concentrated to obtain a yellow-orange oil (2.7 g) that was chromatographed over a Si gel column (72 g) with 20% EtOAc in hexane as eluent. The less polar fractions afforded a mixture of 4-deoxyasbestinin A [**1**] and 4-deoxyasbestinin C [**3**], which was separated by hplc on a Partisil-Si column (5% EtOAc in hexane) to yield pure **3** (colorless oil, 8 mg, 0.003% dry wt) and **1** (colorless oil, 150 mg, 0.034% dry wt). The intermediate polar fractions afforded pure 11-acetoxy-4-eoxyasbestinin D [**4**] (colorless oil, 76 mg, 0.072% dry wt), a mixture of **2** and **4** (126 mg), and a third fraction containing 11-acetoxy-4-deoxyasbestinin B [**2**], a crystalline solid which after recrystallization from MeOH afforded pure white crystals (501 mg, 0.430% dry wt).

PHYSICAL AND SPECTRAL DATA FOR 4-DEOXYASBESTININ A [**1**].—Colorless oil: $[\alpha]^{29}D$ −6.6° (c = 1.60 g/100 ml, $CHCl_3$); ir (neat) 2961, 1731 cm^{-1}; hreims *m/z* $[M]^+$ 390.2718 (2%) ($C_{24}H_{38}O_4$ requires 390.2760), 303 (10), 221 (12), 191 (31), 134 (33), 93 (41), 71 (100); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2.

11-ACETOXY-4-DEOXYASBESTININ B [**2**].—Crystalline solid: mp 150–152°; $[\alpha]^{29}D$ −8.90° (c = 0.34 g/100 ml, $CHCl_3$); ir (KBr) 2956, 1730, 1246, 1238, 1066 cm^{-1}; hreims *m/z* $[M]^+$ 362.2456 (14%) ($C_{22}H_{34}O_4$ requires 362.2448) 303 (20), 174 (61), 134 (74), 105 (88), 93 (100); ^{1}H nmr see Table 1; ^{13}C nmr see Table 2.

4-DEOXYASBESTININ C [**3**].—Colorless oil: $[\alpha]^{29}D -1.2°$ ($c = 0.84$ g/100 ml, $CHCl_3$); ir (neat) 2928, 1730, 1250, 1174, 1089 cm^{-1}; hreims *m/z* $[M]^+$ 390.2765 (12%) ($C_{24}H_{38}O_4$ requires 390.2760), 303 (45), 221 (29), 177 (31), 93 (68), 71 (100); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

11-ACETOXY-4-DEOXYASBESTININ D [**4**].—Colorless oil: $[\alpha]^{29}D -2.29°$ ($c = 1.31$ g/100 ml, $CHCl_3$); ir (neat) 2932, 1738 cm^{-1}; hreims *m/z* $[M]^+$ 362.2439 (3%) ($C_{22}H_{34}O_4$ requires 362.2448) 303 (10), 221 (11), 150 (14), 93 (31), 43 (100); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

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