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Proton Nuclear Magnetic Study of Bistramide A, a new cytotoxic drug isolated from Lissoclinum Bistratum Sluiter

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ABSTRACT

Modern two-dimensional NMR techniques have been used here in order to study the structure of a recently isolated cytotoxic drug, bistramide A. Mass spectroscopy indicated a M_r of 704 corresponding to an apparent molecular formula of $C_{40}H_{68}N_2O_8$. All structural information was obtained from ^1H and ^{13}C NMR. ^1H-^1H and ^1H-^{13}C COSY in combination with relayed ^1H-^1H-^{13}C COSY and ^1H-^{13}C COLOC were used for obtaining all crucial connectivies required for determining the partial structure of this natural product.

Considerable interest with regard to the possible clinical use of small lipophilic molecules possessing high cytotoxic properties and isolated from Tunicate[1-8] prompts us to report isolation and structure of a new compound extracted from *Lissoclinum bistratum* Sluiter named bistramide A.

Interesting biological properties of bistramide A such as neuro and cytotoxicity have been found[9] and we have undertaken the structure elucidation of the drug. Bistramide A failed to give crystals suitable for X-ray diffraction studies. This involves the application of nuclear magnetic resonance spectroscopy in solution using a combination of several two-dimensional NMR techniques for proposal of a structure.

RESULTS

The FAB mass spectrum data indicate a molecular weight of 704. A $C_{40}H_{68}N_2O_8$ formula has been obtained from the high resolution mass spectrum. The chemical ionization spectrum gave a characteristic peak at 705 ($M^+ + 1$) and some fragmentation ions at m/e = 368, 406, 424 which can be analyzed (see below).

The ^1H NMR spectrum (Figure 1) clearly shows the presence of two NH protons at 7.30 ppm and 6.95 ppm and the presence of olefinic protons at 5.20 ppm, 6.15 ppm and 6.90 ppm. The later exhibits a spin-coupling with a coupling constant (J = 15.0 Hz) which unambiguously indicates the trans position of the olefinic protons. From the methyl region, 7 CH_3 have been detected. One of them (δ = 1.91 ppm) is coupled with the olefinic CH proton at 6.90 ppm (J = 6.5 Hz) ; another methyl (δ = 1.25 ppm) is coupled with a CH proton at 4.20 ppm (J = 6.0 Hz). These couplings are corroborated using a decoupling technique by irradiation at 6.90 and 4.20 ppm (not shown). The same technique applied to the other methyl signals exhibits the following couplings : CH_3 (1.26) -CH (2.38) (J = 7.0 Hz) ; CH_3 (0.96) -CH (2.36) (J = 6.5 Hz) ; CH_3 (0.86) -CH (1.92) (J = 7.0 Hz). A sixth CH_3 (1.62) shows a long range coupling with the trans olefinic proton = CH- (5.20) (J = 1.0 Hz). The last CH_3 (0.82) is coupled to a CH which will be identified otherwise (J = 6.5 Hz).

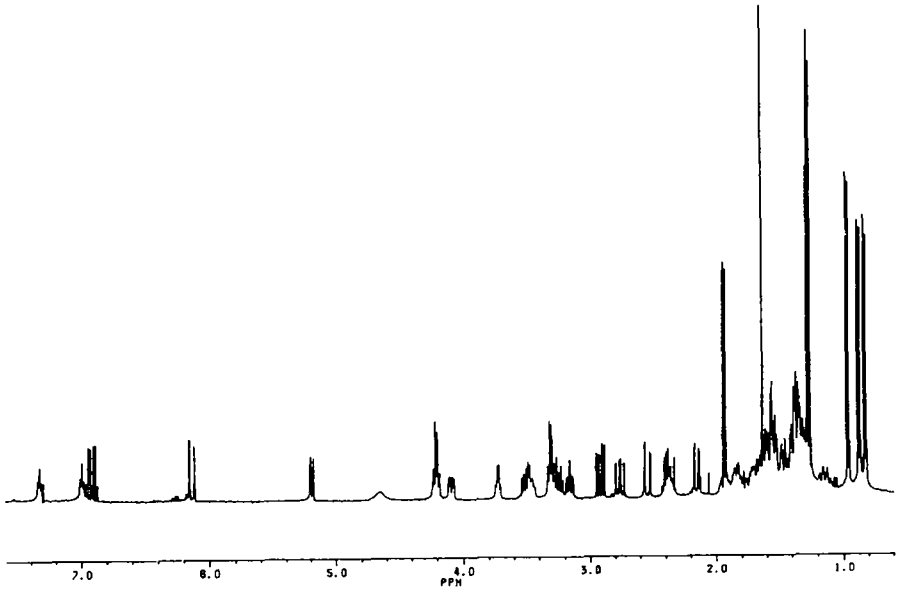

Figure 1. ^1H NMR spectrum of bistramide A

Examination of the proton decoupled ^{13}C spectrum revealed the presence of 40 carbon atoms (Figure 2). The number of protons coupled to a particular carbon was easily obtained by the use of DEPT pulse sequences (Figure 3). Proton coupling constants were obtained through the use of 2D proton carbon heteronuclear J resolved technique (not shown). The experimental data are reported in Table 1. 7 CH_3, 15 CH_2, 13 CH groups were identified. Chemical shifts data unambiguously allowed the determination of one ketone group (δ = 198.89), two carboxamides (δ = 175.14 and 173.42), four olefinic carbons (δ = 144.50, 137.16, 132.07, 131.32), one hemi-acetalic carbon (δ = 95.44) and six CH-O ether groups (δ = 74.82, 74.26, 73.81, 73.26, 69.07, 64.80).

TABLE I

^{13}C ppm	^{1}H ppm	J Hz	Assignment
11.82	1.62	n.o.	CH_3
15.57	1.26	n.o.	CH_3
17.14	0.86	128.6	CH_3
18.02	0.82	125.1	CH_3
18.43	1.91	125.1	CH_3
19.23	1.54 ; 1.83	131.0	CH_2
20.97	0.96	125.1	CH_3
21.75	1.25	127.5	CH_3
25.86	1.55 ; 1.82	124.0	CH_2
26.52	1.33 ; 1.63	132.2	CH_2
27.91	1.46 ; 1.58	125.6	CH_2
30.44	1.36 ; 1.73	124.0	CH_2
30.78	1.41 ; 1.69	129.8	CH_2
31.34	1.13 ; 1.52	125.1	CH_2
31.88	2.36	128.7	CH
32.33	2.15 ; 2.75	127.5	CH_2
33.32	1.92	129.8	CH
33.48	1.30 ; 1.41	120.5	CH_2
34.09	1.29 ; 1.42	127.5	CH_2
34.89	1.29	126.3	CH
35.47	1.38 ; 1.56	126.3	CH_2
36.10	1.45 ; 1.61	124.0	CH_2
39.49	3.30 ; 3.30	138.0	CH_2
43.36	2.38	128.7	CH
44.85	3.24 ; 3.50	138.0	CH_2
45.24	2.53 ; 2.91	125.1	CH_2
64.80	4.20	140.4	CH
69.07	3.45	142.7	CH
73.26	4.195	147.4	CH
73.81	3.72	145.0	CH
74.26	3.15	142.7	CH
74.82	4.06	143.9	CH
131.32	5.20	156.7	CH
132.07	6.15	154.4	CH
144.50	6.90	153.2	CH

n.o. non observed

^{1}H-^{13}C coupling constants

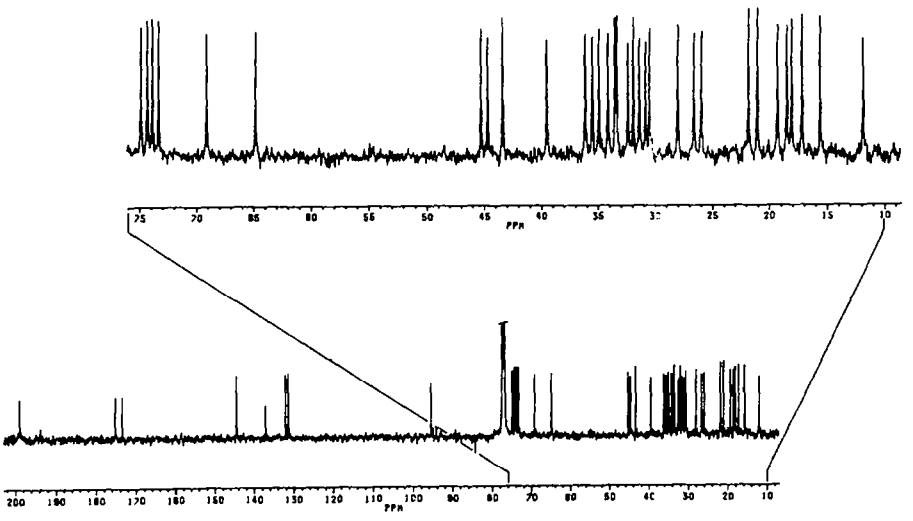

Figure 2. ^{13}C NMR spectrum of bistramide A

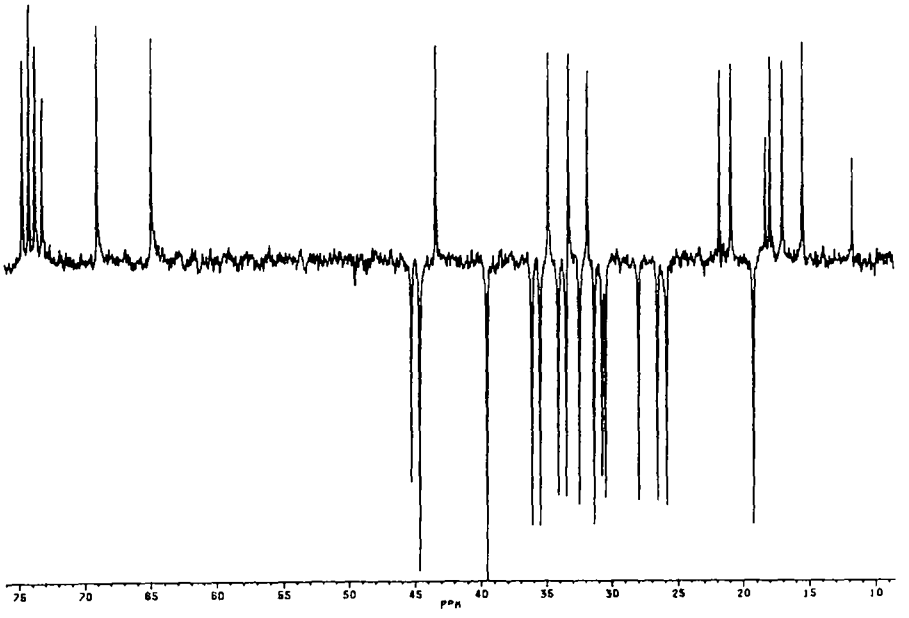

Figure 3. DEPT spectrum (10-75 ppm region) of bistramide A (CH_2 < 0, CH and CH_3 > 0).

The structure was further analyzed by combining 2D NMR pulse sequences. The direct heteronuclear (^{1}H-^{13}C) chemical shift correlation allows simultaneous determination of ^{1}H and ^{13}C chemical shifts for directly bonded $^{13}C^{1}H_n$ units. The correlation map is given in Figure 4 and the results are given in Table 1. A typical COSY-45 experiment was then realized enabling us to obtain the linkage between CH_n units. The full COSY matrix is shown in Figure 5 along with the conventional F_2 projection. All the ^{1}H-^{1}H couplings previously determined by irradiation have been confirmed. A lot of connectivities have been then pointed out. Starting from the NH proton at 6.95 ppm the following chain has been identified : NH (6.95), CH_2 (3.30), CH_2 (1.55, 1.82), CH_2 (1.75, 1.36), CH (3.15), CH_3 (1.29). By the same way starting from the other NH proton at 7.30 ppm another chain could be elaborated : CH_2 (3.50, 3.24), CH (3.72), CH (2.38). Other parts of the molecule can be built on the basis of the COSY observations :

- CH_2 (2.15, 2.75), CH (4.06), CH (1.92), CH_2 (1.63, 1.33)
- CH_2 (1.52, 1.13), CH (3.45)
- CH_2 (2.91, 2.53), CH (4.20), CH_2 (1.69, 1.41)

The whole connectivities are reported in Figure 8, along with the one obtained with a ^{1}H-^{13}C long range correlation (COLOC) which allows the identification of connectivities through $^{2}J_{CH}$ and $^{3}J_{CH}$ couplings (Figure 6).

Among the correlations obtained some key connectivities have to be noticed, linking the carbon chains previously observed :

- C (198.89) of the olefinic ketone group and protons of the methylene H_2 (2.91, 2.53) ;
- C (175.14) of the carboxamide group and H_3 (1.26) ;
- C (173.42) of the carboxamide group and H_2 (2.75, 2.15) ;
- C (95.44) of the hemiacetal group and H (1.46) of methylene at 36.10 (δ_C) ;
- C (34.89) and proton (1.58) of methylene at 27.91 (δ_C) ;
- C (27.91) and H_3 at 0.82 ;
- C (35.47) and H at 1.63 of methylene at 26.52 (δ_C) ;
- C (33.48) and H_3 at 0.96.

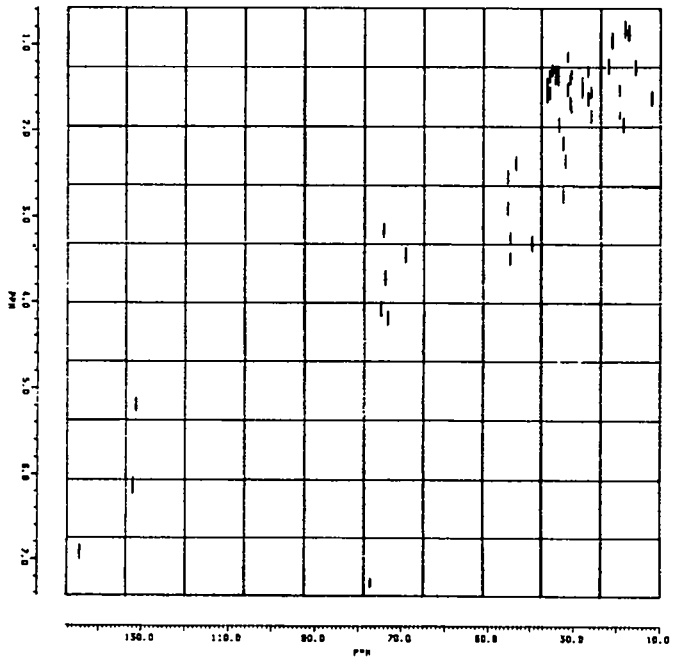

Figure 4. Heteronuclear (^{1}H-^{13}C) correlation spectrum of bistramide A.

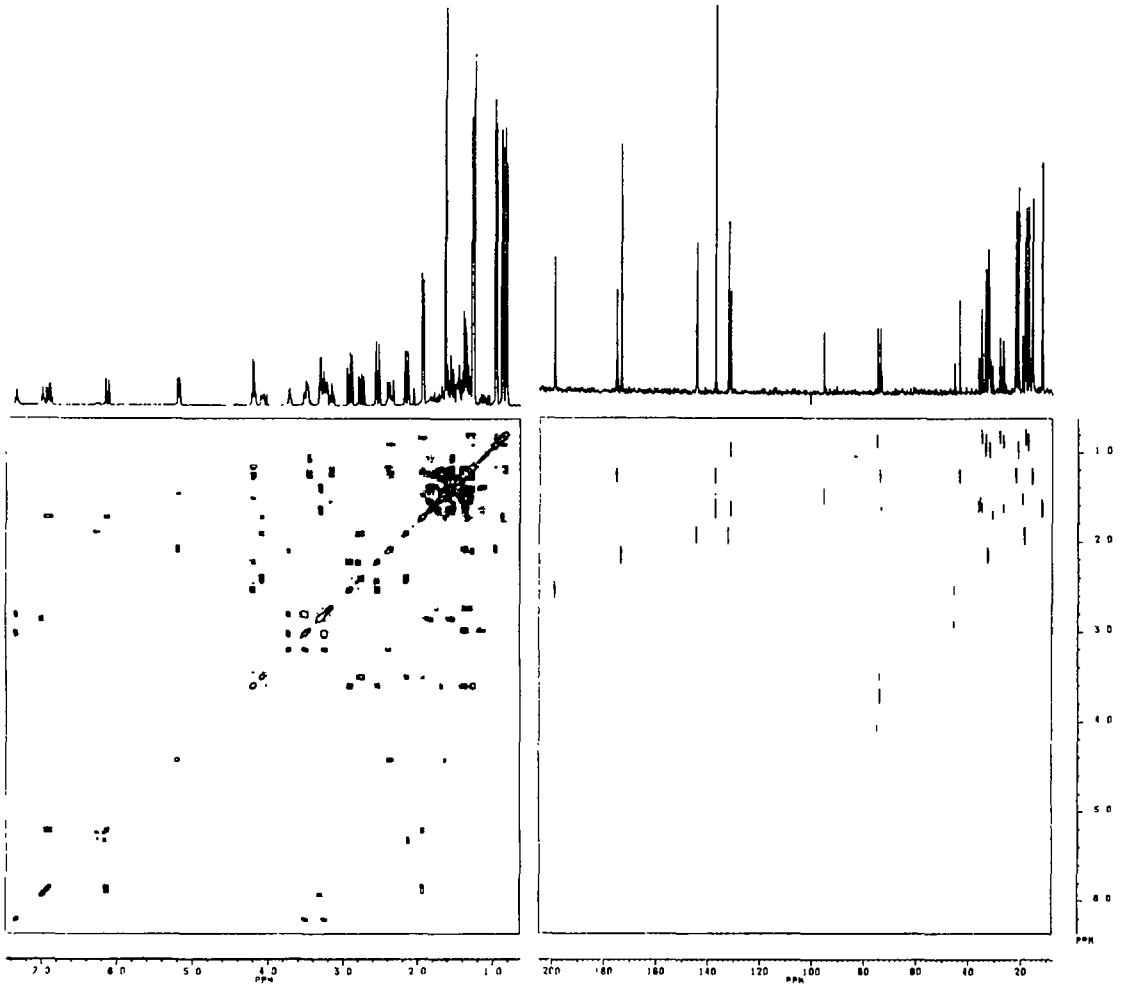

Figure 5. COSY 45 matrix with conventional F_2 projection along one axis.

Figure 6. Heteronuclear (^1H-^{13}C) long range correlation [COLOC] spectrum of bistramide A.

An additional correlation technique was used ; a relay ^1H-^1H-^{13}C transfer which gives correlation (^1H-^{13}C) from distant protons via H-H couplings.

The correlation map is shown in Figure 7 and the connectivities are reported in Figure 8. Some new and important connectivities have to be pointed out :

- C (30.78) and H (1.61) of methylene at 36.10 (δ_C) ;
- C (35.47) and H (1.83) of methylene at 19.23 (δ_C) ;
- C (34.09) and H (3.45) of CH group at 69.07 (δ_C).

The stereochemistry of the olefinic bonds has been determined from measuring ^1H-^1H coupling constants between the olefinic CH(6.15, 6.90, J = 15.0 Hz) and a long range coupling between the olefinic CH(5.20) and the CH_3(1.62, J = 1.0 Hz).

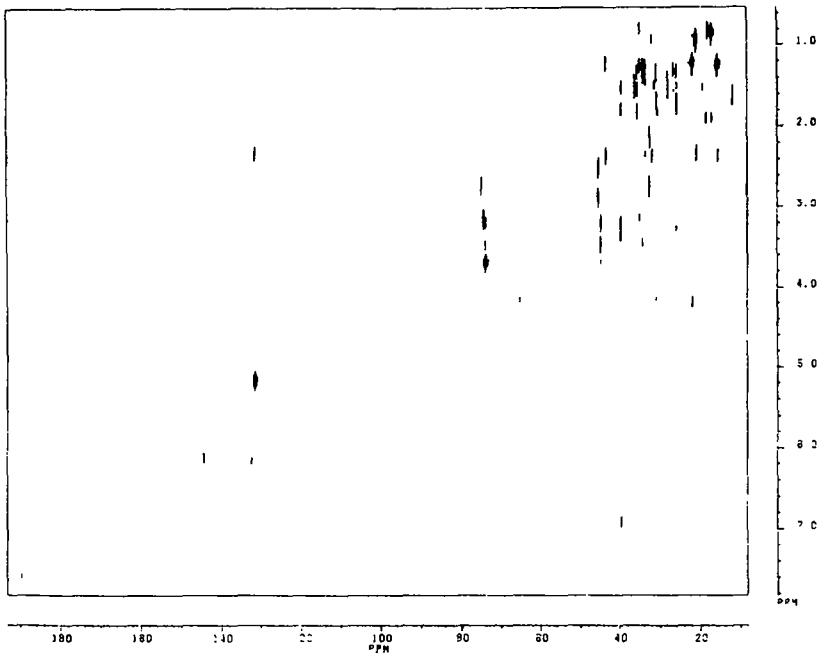

Figure 7. Relayed ^{1}H-^{1}H-^{13}C correlation spectrum of bistramide A.

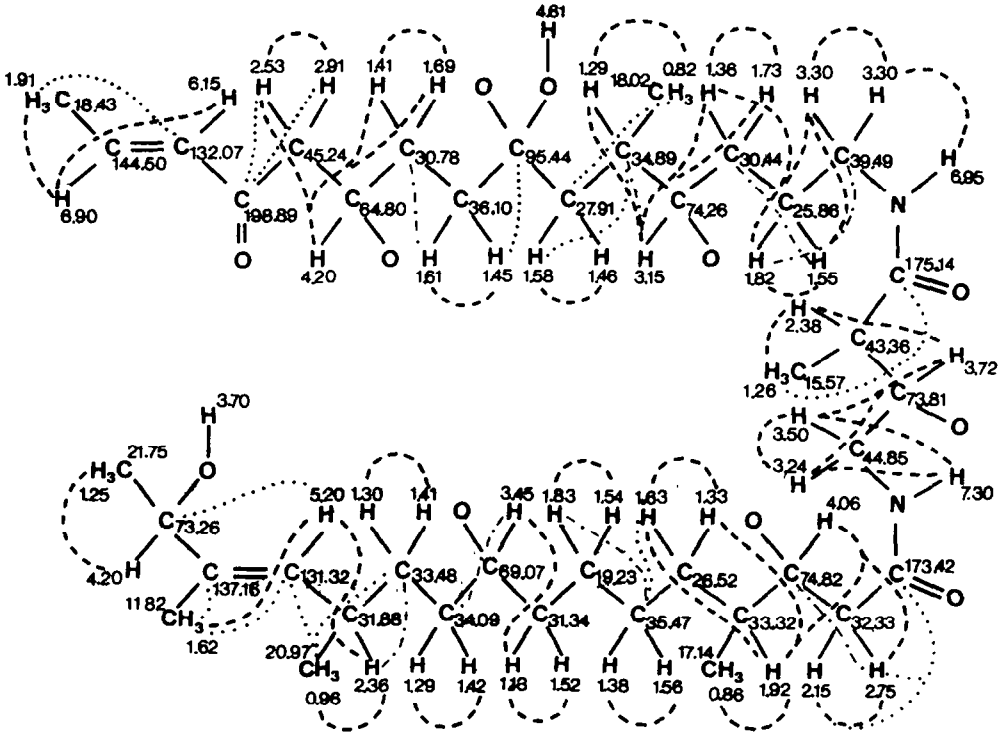

Figure 8. Proposed linear enchainment for bistramide A. Significant correlations have been reported : COSY 45 (--) ; Relayed ^{1}H-^{1}H-^{13}C correlation (-··-) ; COLOC (···).

DISCUSSION

The data obtained by using two-dimensional NMR techniques discussed above are very useful for the determination of the structure. The ^{1}H-^{13}C correlation method clearly identifies the CH_n (n = 1, 2, 3) groups and the ^{1}H-^{1}H correlation COSY experiment is particularly interesting to detect connectivities not directly observable by decoupling.

The connectivities leading to the structure of Figure 8 have been deduced from the conjugate methods COSY, RELAY and COLOC. It is obvious that data obtained by the COLOC technique are not sufficient to confirm all the data recorded by the other 2D techniques but have yielded some deciding informations. Since a linear enchainment is definitely established for bistramide A, the last problem to be solved is an unambiguous bridging of ether-oxyde groups. In an attempt to answer this question a NOESY experiment was performed, showing spatial proximity between some hydrogens. For example, on a two-dimensional NOESY contour map (not reported), NOE cross-peaks were observed between the protons H(2.53), H(2.91) and CH_3(1.25), and between the protons CH(6.15) and CH_3(1.25) and CH(4.20) indicating the close proximity of the two end-chains. In addition, NOEs between the protons (i) H(2.53), H(2.91), H(2.75) and CH_3(1.25), (ii) H(2.75) and CH_3(1.91), (iii) H(2.15) and CH(6.15) indicate the proximity of the C(32.33), H(2.15), H(2.75) methylene to the allylic end chains.

On the other hand during this study another isomer, bistramide C, has been purified and shown by NMR to differ from bistramide A by oxidation of the alcohol borne by one of the allylic chains. This chemical modification is followed by variations in ^{13}C and ^{1}H chemical shifts of this chain ; surprising is the variation of chemical shifts of the other allylic chain and this can be one more argument in favour of the spatial proximity of the two end-chains.

Nevertheless these structural informations are not sufficient by themselves to allow the proposal of a definite three-dimensional structure for bistramide A.

Simultaneously chemical methods were used to degrade the hemiacetalic linkage in the molecule. Unfortunately many degradation products were obtained whatever the method used, unabling us to propose a definite structure for bistramide A.

Though all these experimental results allow the unequivoqual proposal of a linear enchainment for bistramide A (Figure 8), many cyclisations can be envisaged in order to closely relate the allylic end-chains of the molecule.

This work shows the limits of two-dimensional NMR spectroscopy for determining the structure of a new derivative. May be improvements in this technique will help us in a near future to establish the structure of bistramide A.

EXPERIMENTAL

Bistramide A was isolated from *Lissoclinum bistratum* Sluiter (UROCORDATA - Didemnidae) collected near the Ua islet, New Caledonia, in February 1986. The lyophilized powder was extracted with CH_2Cl_2 at ambient temperature. The extract was chromatographed (Prepamatic LC2 JOBIN-YVON) on silica-gel 60 : 70-200 µm and 40-70 µm with EtOAc and on Lichroprep^{R} diol (Merck) : 25-40 µm with n-hexane/EtOAc (5:5), leading to 4.8 g of bistramide A (0.16 % dry weight). The purity of bistramide A has been confirmed by HPLC (LDC Constametric III, column Hibar Lichrosorb^{R} diol - 5 µm Merck, n-hexane/EtOAc 5:5).

Mass spectrometry.

Fast atom bombardment mass spectra of bistramide A were obtained on a Kratos MS-50 double focusing mass spectrometer. The samples were dissolved in thioglycerol and a small drop of the sample solution was placed on the copper target of the FAB direct insertion probe. The sample was bombarded with 8 keV xenon atoms, and the ions produced were accelerated through 8 kV.

Relayed ^{1}H-^{1}H-^{13}C correlation spectroscopy

This experiment[15] was realized with the same parameters as for H,C-COSY. The mixing time to optimize modulations by H-H coupling was varied from 10 to 25 ms. The maximum data were collected from the matrix realized with a 10 ms mixing time.

Heteronuclear (^{1}H-^{13}C) long range correlation spectroscopy (COLOC)[16]

The parameters were those of the heteronuclear (^{1}H-^{13}C) correlation spectrum .The optimum D_2 delay was 50 ms.

Nuclear Overhauser Effect spectroscopy (NOESY)

The 2D NOE experiments were performed by using the pulse sequence of States[17]. NOESY data sets (512 x 4K) with a mixing time of 250 ms were collected.

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