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OKADAIC ACID A PROTON AND CARBON NMR STUDY

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Abstract Okadaic acid is an extremely interesting toxin responsible for diarrhetic shellfish poisoning (DSP) This interest is due not only to its fascinating structure but also to its unknown biosynthetic origin In this paper we report on the full NMR study of this molecule, including the appropriate 2D homo and heteronuclear NMR experiments, neccesary to carried out its biosynthetic study

Prorocentrum lima is one of the dinoflagellates that produce okadaic acid (OA), a toxin responsible for food poisoning occurring in the Rías Gallegas (Galicia, NW Spain) OA and its derivatives known as dinophysistoxins (DTXs), a group of lipid soluble toxins responsible for diarrhetic shellfish poisoning (DSP),[1] have attracted considerable attention since their isolation and structural elucidation They are characterized by possessing a polyether structure, this structural feature being common not only to other groups of dinoflagellate toxins such as the brevetoxins (BTXs)[2] and ciguatoxin,[3] but also to polyether antibiotics[4] like monensin A, naransin, lasalocid, etc , isolated from terrestrial microorganisms

Without a doubt, one of the most intriguing problems related with these toxins is their biosynthetic origin Thus, the biosynthesis of BTXs has only recently been reported,[5] showing an unusual involvement of the citric acid cycle in comparison with the well-established studies of the related terrestrial polyether in which the intact incorporation of the precursors has been observed This fact has been proposed as the more general difference between terrestrial and marine organisms However, the structures of BTXs are characterized by the presence of medium-size ethereal rings transfused in a ladder-like manner, while the terrestrial organisms possess either isolated or spiro oxane and oxolane ethereal rings Taking this fact into account and in order to explore the above proposal, the biosynthetic study of OA and its derivatives has been considered interesting because, unlike the BTXs, their structures resemble those of the terrestrial polyethers

The first problem to be resolved in any biosynthetic study is to assign all proton and carbon

NMR chemical shifts of the metabolite under study In this paper, we report on the NMR study of OA

Okadaic acid was obtained from the unialgal cultures of *Prorocentrum lima* in accordance with the chromatographic process described in the experimental part. During this process, we obtained samples of OA complexed to a metallic cation These samples could not be distinguished from pure okadaic acid during the chromatography because their chromatographic behaviour under the conditions used were identical However, the two samples showed significantly different ^{1}H-NMR spectra· the metallic complex showed a much poorer resolution, undergoing individual chemical shift differences (Fig 1) Treatment of this sample with EDTA disodium salt yielded pure free okadaic acid.

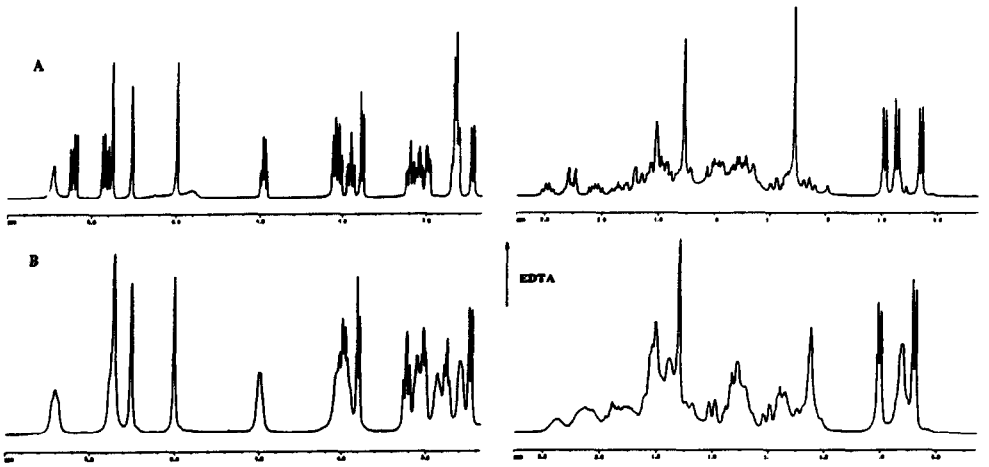

Figure 1 Comparison of the ^{1}H-NMR (600 MHz) spectra of Okadaic Acid (A) and complexed OA (B) in $CDCl_3$

Proton NMR study of Okadaic Acid

Configurational and conformational features of okadaic acid have been derived from scalar and dipolar coupling connectivities extracted from 2D-NMR experiments, obtained from the two samples, the free acid and its complex with a metallic cation Thus, COSY,[6] DQ-COSY,[7] NOESY,[9] ROESY,[10] conventional and long range ^{1}H-^{13}C (HMQC)[11] were run on okadaic acid, while TOCSY,[8] COSY, conventional and long range ^{1}H-^{13}C COSY (HMQC) experiments were run on the complex

For the NMR study, the presence in the molecule of four quaternary centres at C-8, C-19, C-25 and C-34, conveniently broke it into five separate directly proton-coupled spin systems, thus simplifying the analysis of the 2D-NMR spectrum Thus, OA was divided into five regions and the proton assignments will be described in this manner

Region I

The proton assignments in region I may be conveniently started from H-4 (δ 3 96), which is the only α-methine to an oxygen atom in the molecule coupled to two methylene groups, the H-3s (δ 2 12 and 1 62) and H-5s (δ 1 72 and 1 31) The latter protons were coupled with H-6s (δ 1 83 and

1 79) and these in turn to H-7 (δ 3 34), terminating the system of coupled protons in this region with the presence of a quaternary centre at C-8. The diastereotopicities at carbons C-3, C-5 and C-6 were established by analysing the observed NOE(ROE) connectivities Thus, H-4 showed strong ROE connections with the protons H-3 (δ 2.12), H-5 (δ 1 31) and H-6 (δ 1 79), while it had a weak connection with H-3 (δ 1 62) Moreover, a NOE connectivity was observed between H-3 (δ 2 12) and H-5 (δ 1 31) (Fig 2) The patterns shown by the H-4 signal, two large (9Hz) and two small (3Hz) couplings, confirmed the axial orientation of the corresponding bond.

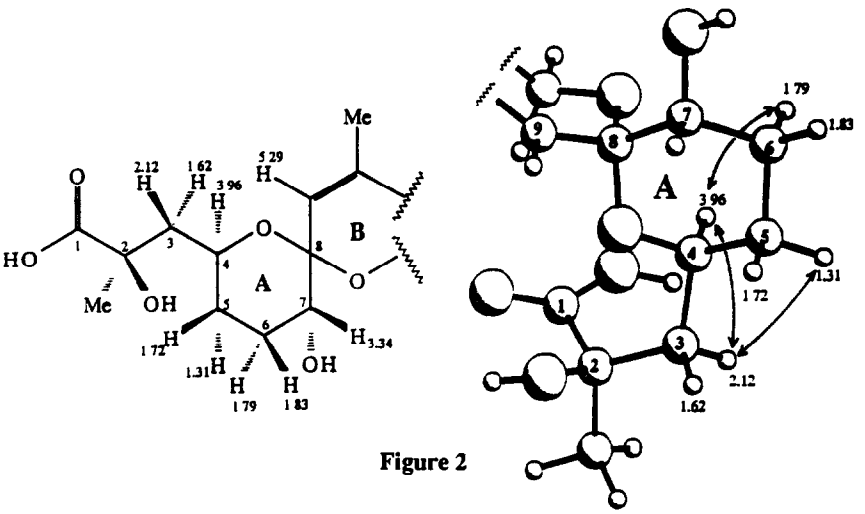

Figure 2

Region II

The pair of olefinic protons H-14 and H-15 was a good starting point for the assignment of region II H-15 (δ 5 42) was coupled to the allylic α to oxygen H-16 (δ 4 51) Further coupling of H-16 to H-17s (δ 1 54 and 2 14) and of H-17s to H-18s (δ 1 80 and 2 04) was observed Going towards the quaternary olefinic carbon C-10, H-14 (δ 5 63) was coupled to H-13 (δ 2 21), this one to H-12 (δ 3 35) the latter being coupled to the pair H-11s (δ 1 91 and 1 87)

The configurational assignments of the acyclic moiety of this region were established as follows Starting from H-14, three cross-peaks could be labelled with confidence in the NOE(ROE) spectra, H-14 / H-12, H-14 / H-16 and H-14 / Me-13 (δ 0 97) Likewise, H-15 was shown to be connected with H-13 and the methyl group Me-13 In this case, the diastereotopicity of the methylene protons C(11), C(17) and C(18) was again established by the observed NOE(ROE) connectivities Thus, H-13 and H-11 (δ 1 87) as well as H-16 and H-18 (δ 2 04) were shown to be connected Moreover, a high intensity cross-peak was observed in the ROESY experiment between H-16 and H-17 (δ 2 14) establishing that both were on the same face of the molecule (Fig 3)

The H-12 signal showed two large couplings (9Hz) and one small (4Hz) in agreement with the conformation in Fig 3 Finally, the H-11s signals and the methyl group (δ 1 73) were connected to the olefinic proton H-9 by virtue of the allylic couplings

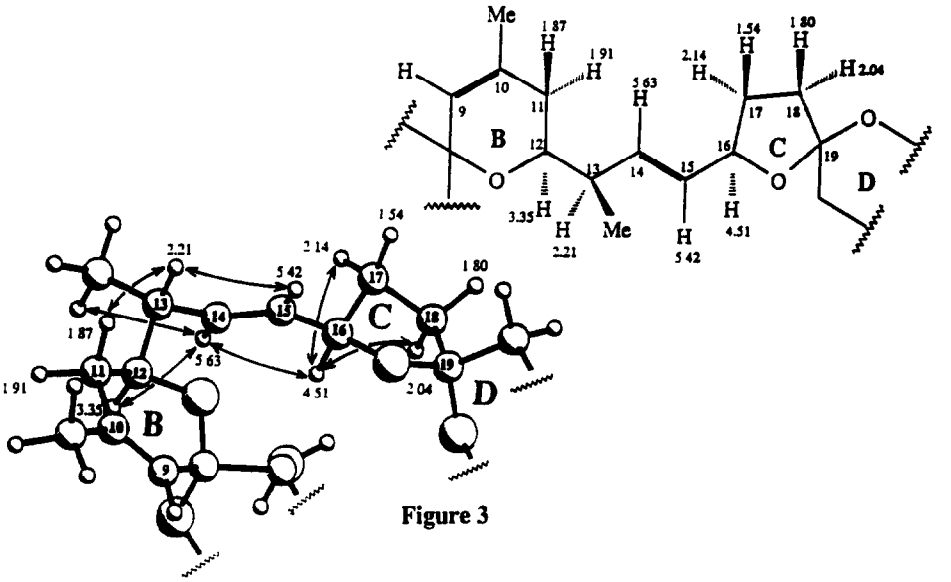

Figure 3

Region III

The connectivities for H-24 through H-20 protons began with the identification of H-24 (δ 4 07) which showed a long-range coupling with the protons of the exocyclic double bond H-42 (δ 5 02 and 5 39) The connectivities towards the quaternary centre at C-19 showed vicinal couplings between H-24 (δ 4 07) and H-23 (δ 3 35), the latter in turn was coupled to H-22 (δ 3 57), followed by the coupling of this proton to H-21s (δ 1 81 and 1 72) The chemical shifts for H-20s could not be clearly established as their cross-peaks were close to the diagonal The β-orientations of protons H-24 and H-22 were confirmed by a NOE connectivity between them A strong ROE connectivity was also observed between H-21 (δ 1 81) and H-22 which solved the assignments for H-21s (Fig 4) The observed couplings, $J_{23\,24}$ 10Hz, $J_{22\,23}$ 10Hz, $J_{21\,22}$ 10Hz (δ_{21} 1 72) and $J_{21\,22}$ 4Hz (δ_{21} 1 81) were in full agreement with the stereochemistry of this region, as illustrated in Fig 4

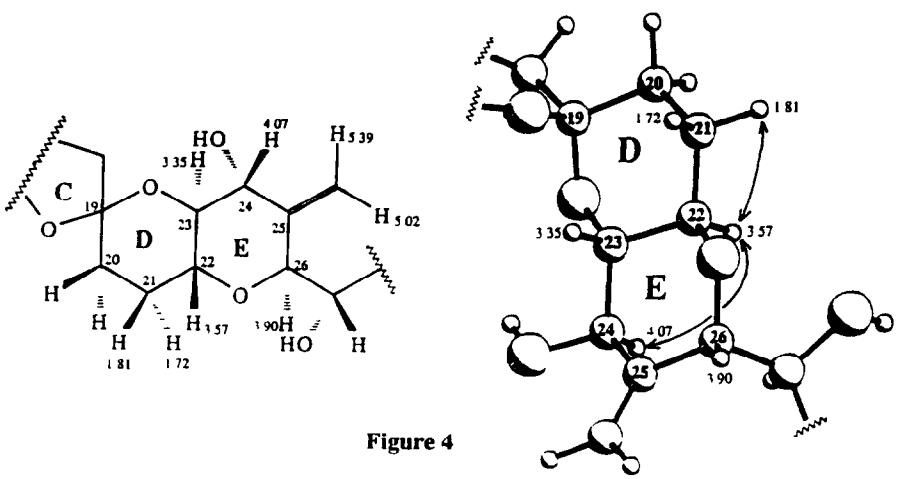

Figure 4

Region IV

Here again, the connectivities of this region begin with a long range coupling between the olefinic proton H-42 (δ 5 02) and H-26 (δ 3 90) All the vicinal and geminal proton connectivities can be clearly spotted in the COSY map from H-26 to the pair H-33, thus unambiguously establishing their assignments

The diastereotopicity of H-28s was established by the NOE(ROE) connectivities observed between these protons and H-26 and H-30 Thus, strong intensity cross-peaks were observed between the H-28 (δ 0 95) and H-30, while the other H-28 (δ 1 28) was connected with H-26 Moreover, a weak intensity cross-peak was observed between H-28 (δ 0 95) / H-26 and between H-28 (δ 1 28) / H-30 The conformation of the linear moiety of this region was totally defined by the connectivity observed between the H-27 and the methyl group at carbon C-29 Regarding the heterocyclic moiety, the connectivities observed between H-30 and the H-32 (δ 1 96) as well as that observed between the H-33 (δ 1 34) and the methyl group at C-31, define the diastereotopicities of the methylene groups C-32 and C-33 (Fig 5) Observed vicinal couplings, $J_{26\,27}$ 9Hz, $J_{27\,28}$ 10Hz (δ_{28} 1 28), $J_{27\,28}$ (δ_{28} 0 95), $J_{29\,30}$ 10Hz and $J_{30\,31}$ 2Hz, were in full agreement with the above conclusions

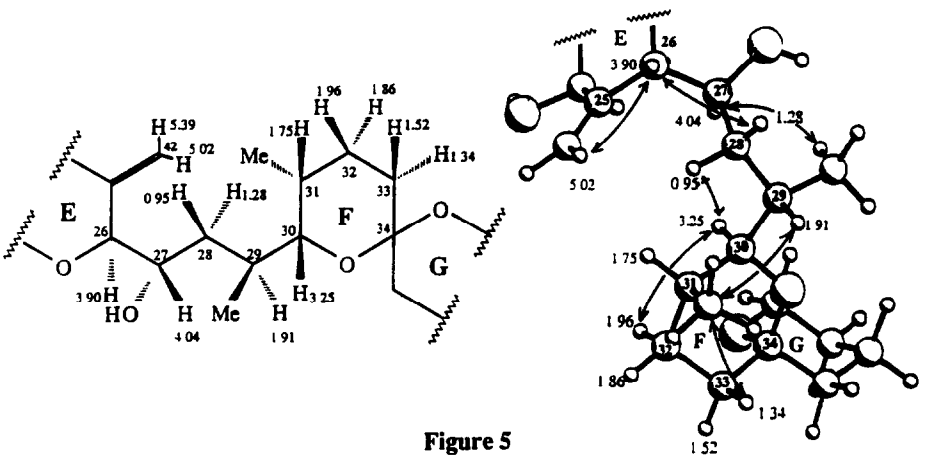

Figure 5

Region V

The starting point for the proton assignments in this region was obviously the methylene group α to oxygen H-38s (δ 3 62 and 3 53) Both protons were coupled to H-37s (δ 1 51 and 1 84) These in turn were coupled to the pair H-36s (δ 1 61 and 1 39) Unfortunately, as in the case of H-20s, we were unable to find the correlations between these protons and those bonded to C-35

The NOE(ROE) connectivities observed between H-38 (δ 3 62) and H-30 as well as with Me-29 established the diastereotopicity of H-38s protons Likewise, a strong ROE connectivity was observed between H-38 (δ 3 62) and H-37 (δ 1 51) establishing that both were on the same face of the molecule (Fig 6) Observed couplings confirmed that H-38 (δ 3 53) and H-38' (δ 3 62) were, respectively in equatorial and axial orientations

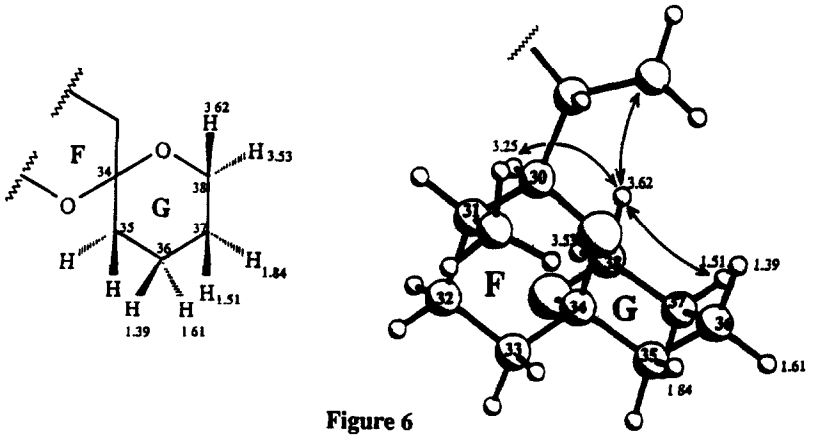

Figure 6

These results definitively established that the conformation of OA in solution is identical with that obtained by X-ray diffraction for this molecule in the solid state.

Carbon NMR study of Okadaic Acid.

After the proton assignments the next step was to assign the carbon resonances to individual carbon atoms in OA. The first step was to identify the multiplicity of each carbon peak Methyl, methylene and methine resonances were identified by the DEPT technique Carbon nuclei C-1, C-42 and C-38 were readily identified on the basis of their multiplicity Thus, C-38 was the only methylene next to an oxygen atom, C-42 was the only olefinic carbon with triplet multiplicity and finally, C-1 could be recognized by its characteristic downfield shift

To extend the carbon assignments further, ^{1}H-^{13}C COSY(HMQC) experiment for one bond coupling was performed (Fig.7) This experiment, together with the DEPT, resulted in the unambiguous assignment of 33 carbons. The remainder could not be assigned by using the conventional HMQC technique, either because the carbons involved were quaternary centres or because their corresponding proton chemical shifts could not be assigned This was the case with the ketalic carbons C-34, C-8, C-19, the quaternary olefinic carbons C-10 and C-25, and the quaternary carbon C-2. In order to resolve this point a long-range HMQC experiment was performed to detect ^{1}H-^{13}C nuclei correlated by scalar coupling through two and three bonds Fig 8 shows this experiment performed on the complexed OA, giving all the necessary correlations Thus, both the long-range correlation between C-8 and the olefinic proton H-9, and that between C-34 and the H-38's methylene protons were observed Thus, the remaining ketalic carbon must be C-19 Likewise, a long-range correlation was observed between carbon C-10 and the only vinylic methyl group present in the molecule, and therefore the remaining quaternary olefinic carbon must be C-25

The still unassigned methylene carbon signals centred at δ 25 4 and 26 4, must correspond to carbons C-20 and C-35 The long-range correlation observed between the signal at δ 25 4 and the proton H-36 defines not only the assignments of the carbon nuclei but also those of the bonded protons

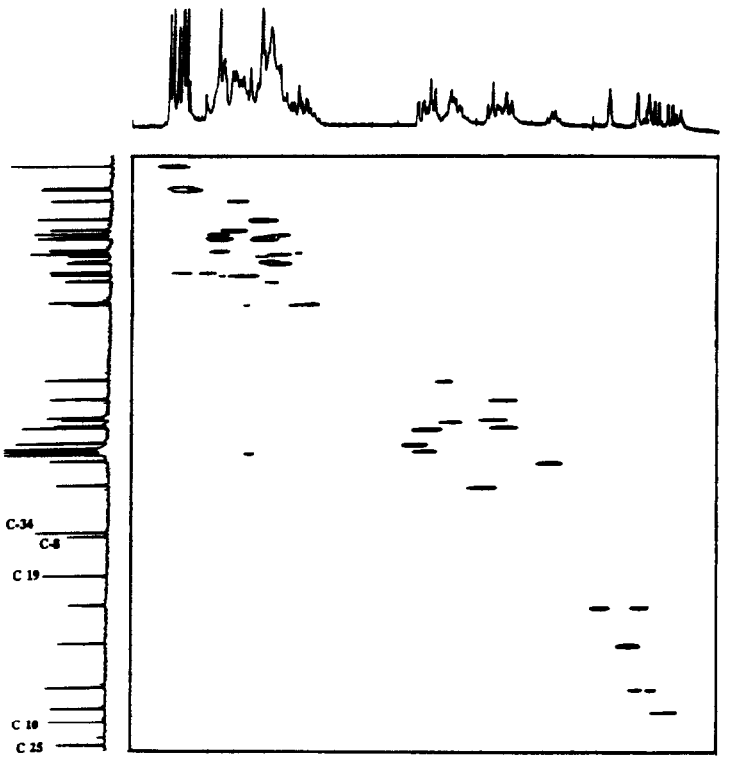

Figure 7 2D-COSY(HMQC) of Okadaic Acid in $CDCl_3$ (200 MHz)

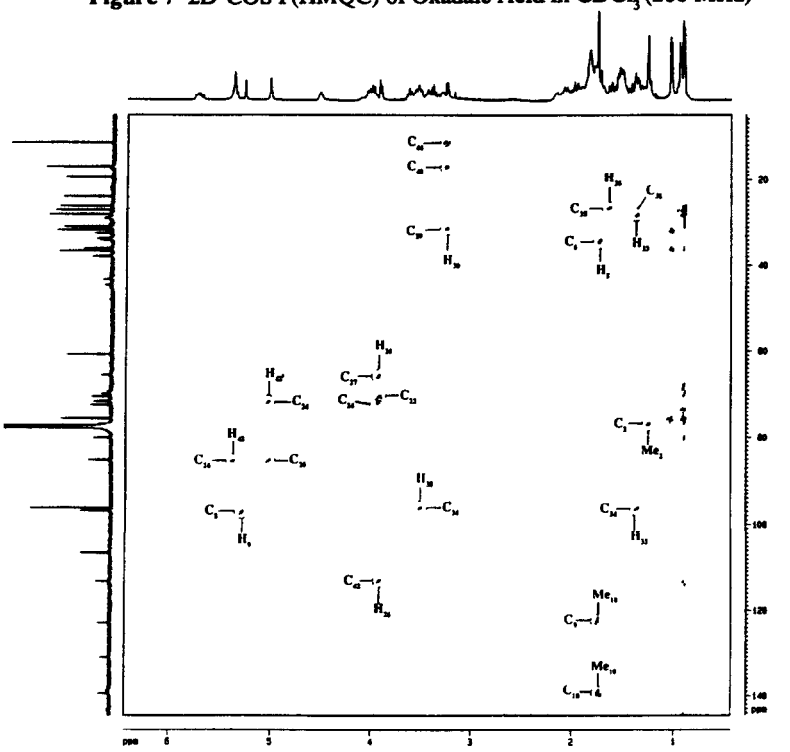

Figure 8 Long range 2D-COSY(HMQC) of complexed OA (500 MHz, J=10 Hz)

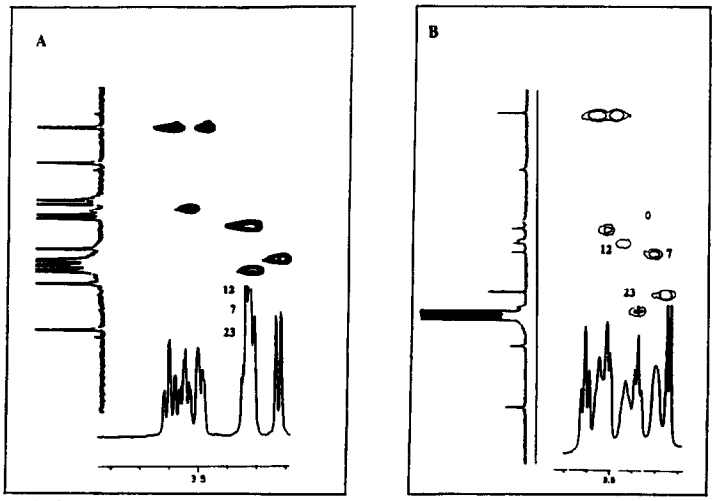

Figure 9 Comparison of selected regions in the 2D-NMR HMQC spectra of Okadaic Acid (A) and complexed OA (B)

A singular situation was observed for carbons C-7, C-12 and C-23 because their proton signals in the free OA sample were overlapped, and it was thus impossible to assign their carbon chemical shifts However, in the proton NMR spectrum of the complex, their chemical shifts were distinguishable and were resolved in the single bond HMQC experiment as shown in the Fig 9 These experiments provided the basis for assigning a δ value of 76.5 ppm to the C-23 carbon in the free acid, while those for C-7 and C-12 must be centred at δ 71 5 Table I gives the full proton and carbon assignments for OA The comparison of these chemical shift values with those obtained for its complex with a metallic cation (see experimental part) showed that the more likely position for the cation to be attached was around the C-14, C-15 double bond

Table I ^{13}C and ^{1}H-NMR ($CDCl_3$) of Okadaic Acid

C	δ	H	δ	C	δ	H	δ	C	δ	H	δ
1	176 7			**17**	30 6	**17**	2 14	**32**	26 6	**32**	1 96
2	77 2					**17'**	1 54			**32'**	1 86
3	42 6	**3**	2 12	**18**	37 3	**18**	1 80	**33**	30 3	**33**	1 52
		3'	1 62			**18'**	2 04			**33'**	1 34
4	69 3	**4**	3 96	**19**	105 7			**34**	95 6		
5	31 6	**5**	1 72	**20**	26 4	**20**	1 32	**35**	25 4	**35**	1 31
		5'	1 31			**20'**	1 47			**35'**	1 48
6	32 8	**6**	1 79	**21**	27 1	**21**	1 81	**36**	35 9	**36**	1 39
		6'	1 83			**21'**	1 72			**36'**	1 61
7	71 5	**7**	3 34	**22**	69 7	**22**	3 57	**37**	18 7	**37**	1 84
8	96 5			**23**	76 5	**23**	3 35			**37'**	1 51
9	121 5	**9**	5 29	**24**	71 1	**24**	4 07	**38**	60 3	**38**	3 62
10	139 4			**25**	144 7					**38'**	3 53
11	33 2	**11**	1 87	**26**	84 9	**26**	3 90	**39(Me-2)**	27 3	**39**	1 36
		11'	1 91	**27**	64 7	**27**	4 04	**40(Me-10)**	23 1	**40**	1 73
12	71 5	**12**	3 35	**28**	35 3	**28**	0 95	**41(Me-13)**	15 9	**41**	0 97
13	42 2	**13**	2 21			**28'**	1 28	**42**	112 5	**42**	5 39
14	136 3	**14**	5 63	**29**	31 1	**29**	1 91			**42'**	5 02
15	131 4	**15**	5 42	**30**	75 0	**30**	3 25	**43(Me-29)**	16 2	**43**	1 01
16	79 1	**16**	4 51	**31**	27 4	**31**	1 75	**44(Me-31)**	10 7	**44**	0 88

EXPERIMENTAL PART

Culture, extraction and isolation procedures.

A typical culture experiment involved inoculation of 20 L of a Guillard K medium with 10 L of *Prorocentrum lima* culture This culture was allowed to grow in a 40 L tank, with constant white fluorescent illumination at 25°C for three weeks After a period of culture, the *P lima* cells were harvested by continuous centrifugation at 7 000 r.p.m. The cells were sonicated and extracted with diethyl ether and chloroform-methanol (1 1) The combined organic solutions were washed with water and the solvent evaporated The resultant organic extract was chromatographed by gel filtration (Sephadex LH-20 column), flash chromatography on a silica gel 60 column, reverse phase medium pressure chromatography on a Lichroprep RP-8 column and the final purification was achieved by μ-bondapack HPLC chromatography All the chromatographies were followed by tlc

Okadaic Acid: $[\alpha]_D^{25}$=+16 8° (c 0 083, $CHCl_3$) All the physical and spectroscopical data were in agreement with those published for okadaic acid[1]

Complexed Okadaic Acid: NMR($CDCl_3$) ^1H (δ) 5.74 (1H), 5 38 (2H), 5 28 (1H), 5 03 (1H), 4 52 (1H), 4 07 (1H), 4 01 (1H), 3 98 (1H), 3 91 (1H), 3.65 (1H), 3.58 (1H), 3 53 (1H), 3 47 (1H), 3 40 (1H), 3 32 (1H), 3 28 (1H), 2 17 (1H), 2 08 (1H), 2 06 (1H), 2 05 (1H), 1 98 (1H), 1 95 (1H), 1.91 (1H), 1 85 (1H), 1 84 (1H), 1 83 (2H), 1 82 (1H), 1 80 (1H), 1 79 (1H), 1.78 (1H), 1 76 (1H), 1 74 (3H), 1 72 (1H), 1 61 (1H), 1 56 (1H), 1 55 (1H), 1 54 (1H), 1 53 (1H), 1 49 (2H), 1 41 (1H), 1 37 (1H), 1 36 (2H), 1 34 (1H), 1 27 (4H), 1 03 (3H), 0 93 (4H), 0 89 (3H) ^{13}C (δ) 176.7 (s), 145 7 (s), 139 4 (s), 138 7 (d), 131 0 (d), 122 8 (d), 113 2 (t), 106 5 (s), 96 9 (s), 96.3 (s), 85 3 (d), 80 2 (d), 77 2 (s), 76 6 (d), 75 8 (d), 72.5 (d), 71.8 (d), 71 6 (d), 70 6 (d), 69 9 (d), 65 7 (d), 61 0 (t), 44 5 (t), 43 3 (d), 38 0 (t), 36 6 (t), 36.0 (t), 34 0 (t), 33 7 (t), 32 5 (t), 31 7 (d), 31 3 (t), 31 0 (t), 29 0 (q), 28 1 (t), 28 1 (d), 27 0 (t) 27 0 (t), 26 1 (t), 23 9 (q), 19 4 (t), 16 9 (q), 16 7 (q), 11.3 (q)

Sample and spectra

Samples for NMR studies were prepared by dissolving OA in 99 98% ($CDCl_3$) Sample concentration was 60 mM and TMS was used as internal reference NMR spectra were acquired using Bruker WP-200 SY (200 MHz), WM-360 (360 MHz), AMX-500 (500 MHz) and AMX-600 (600 MHz) spectrometers Homonuclear COSY, DQF-COSY, TOCSY, NOESY and ROESY and heteronuclear ^1H-^{13}C (HMQC) data, tuned to direct and long range couplings, were obtained.

COSY spectra were recorded in the phase-sensitive mode using the time-proportional phase incrementation mode. The carrier frequency was placed in the centres of the spectrum Data sets consisting of 512 t_1 increments, a variable number of scans/t_1 and 2048 data points in t_2 were collected Prior to Fourier transformation, the 2D data matrix was multiplied in both dimensions with an unshifted sine bell window function and zero-filled up to 2K and 4K in the f_1 and f_2 dimensions, respectively Quadrature detection and a relaxation delay of 1s were used in all cases All homonuclear 2D experiments were acquired under similar conditions TOCSY spectra were recorded with a spin

lock period of 20 ms The mixing time in the NOESY spectra was 300 ms For the ROESY experiment, the mixing time and the radiofrequency field strength were 300 ms and 6 KHz, respectively

Two-dimensional ^{1}H-^{13}C (HMOC) experiments were carried out using polarization transfer from ^{1}H to ^{13}C via J (direct coupling and long-range) on samples with ^{13}C natural abundance. The delays were selected so as to match the respective single bond coupling 125 Hz and long range 10 Hz for one and the other experiment. Broad band decoupling was carried out during acquisition. The offset along the carbon axis was set at the centre such that the spectral width was 200 ppm. t_1 was incremented 512 times at regular intervals The time domain data points were 2048 along the t_2 axis.

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