Letters

Self-Association of Okadaic Acid upon Complexation with Potassium Ion

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Abstract: Okadaic acid (OA) is a toxin responsible for diarrhetic shellfish poisoning and is an extremely useful tool for studying processes that are regulated by phosphorylation, although the exact mechanism of action is still undetermined. We report on a study that proved the existence of OA in an unusual dimeric form when complexed with potassium ion. The proposed structure of this dimer is based on spectroscopic and conformational studies.

Okadaic acid (OA; Figure 1) is the first example of a new group of polyether toxins produced by dinoflagellates,[1] which are responsible for the natural phenomenon known as diarrhetic shellfish poisoning (DSP). This is a form of gastroenteritis that results from eating shellfish, which is a serious problem to public health and to the shellfish industry.[2] In 1987, it was reported that okadaic acid potently and specifically inhibits protein serine/threonine phosphatases types 1 (PP-1) and 2 (PP-2A) with an IC_{50} of 20 and 0.2 nM, respectively.[3] Subsequently, OA has also been shown to be a powerful tumor promoter.[4] These properties have made this toxin a valuable research tool for examining phosphatase-mediated processes and interactions in vivo.[5]

OA is isolated from marine dinoflagellate *Prorocentrum lima* culture in a complexed form that is important to the pharmacological activity of this toxin. Briefly, in a previous study it was shown that the response of smooth muscle to OA is highly depressed in a K^+-free medium, while the complexed form induced a response that is virtually identical and independent of the presence or absence of the cation in the media.[6] These results demonstrated that potassium ions play an important role in OA activity. Therefore, we decided to study the structure of this complex.

The complex existence was initially noted by the different ^{1}H NMR spectra characteristic of each species (Figure 2).[7] However, only one solid-state structure of an *o*-bromobenzyl ester of OA and solution structures in $CDCl_3$, $CDCl_3/CD_3OD$, and $NaOD/D_2O$ of the uncomplexed form of OA have been published until now.[8,9] Despite of the different experimental conditions used in these studies, only minor conformational changes were observed. Here, we report the structure of a K^+-okadaic acid complex (OAC) based on spectroscopic and conformational studies.

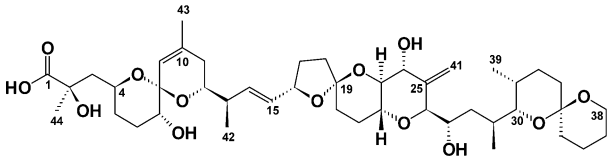

Figure 1. Okadaic acid (OA).

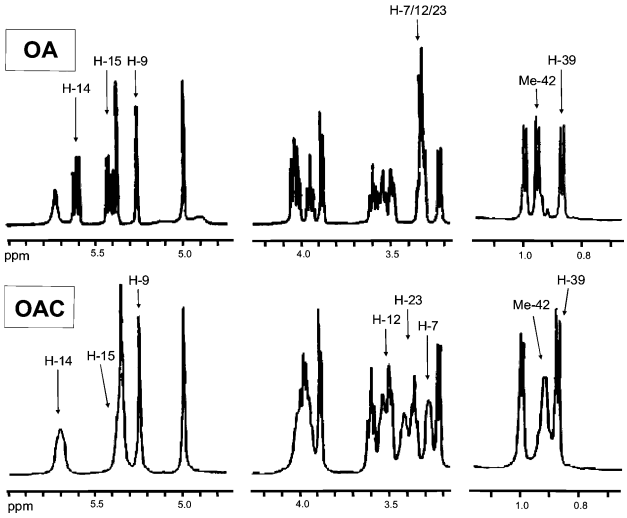

Figure 2. Selected ^{1}H NMR (600 MHz) spectra slices of OA and OAC. It can be seen how the signals corresponding to H-12, H-14, H-15, H-23, and the methyl Me-42 change their positions.

To widen our understanding of the mechanisms by which the complexed form modulates the activity of this toxin, NMR studies were undertaken to determine the structure of the OAC in solution. To be sure of the cation identity in the OAC sample used for NMR, we prepared the complex. For this purpose, a natural OAC sample, isolated in our laboratory from *P. lima*, was decomplexed using EDTA. Then a KCl saturated solution of $MeOH/H_2O$ (85:15) was used to dissolve OA and passed through a Sep-pak C-18 cartridge, eluting with MeOH. Finally, the solvent was evaporated and the sample was dissolved in $CDCl_3$. NOESY experiments using a 400 MHz NMR spectrometer (^{1}H frequency) at 298 K in $CDCl_3$ were carried out with OA and OAC samples. Interestingly, the NOESY spectrum of OAC showed no cross-correlated peaks, in contrast with OA, suggesting that $\omega\tau_c \approx 1.1$ for OAC but not for OA.[10] As expected, ROESY spectra of OA and OAC showed cross-peaks. These data suggest that the molecular weight of OAC may be about 1500 Da, providing a possibility that OAC may comprise two OA units.

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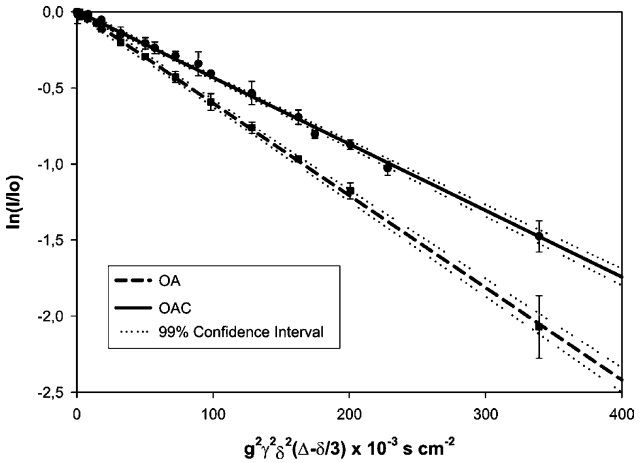

Figure 3. Semilogarithmic plot of the linear regression of the decay of the signal intensity versus the strength of the pulse field gradients applied in the diffusion experiments.[16] Error bars represent one standard deviation from the mean.

To examine the issue of self-association, we measured the translational diffusion coefficients, *D*, for OA and OAC using high-resolution diffusion-ordered spectroscopy (HR-DOSY).[11] Values of *D* were obtained at 283 K with a spinning sample tube to avoid thermal convection problems, and samples with the same concentration were used to avoid differences in viscosity (Figure 3).[12] Each *D* value was determined from four series of 12 one-dimensional PFG-STE experiments[13,14] in which we measured the decay of the intensity of H-9 and H-39 signals. *D* values of $(5.77 \pm 0.05) \times 10^{-6}$ cm^2/s for OA and $(4.47 \pm 0.05) \times 10^{-6}$ cm^2/s for OAC were obtained. The Stokes−Einstein equation was used to relate *D* to the apparent molecular weight M_{app}: $(D_1/D_2)^3 = M_{2app}/M_{1app}$.[15] The result obtained using the *D* values noted above for OA and OAC was $M_{app}(OAC)/M_{app}(OA) = 2.1$, thus confirming that OAC in $CDCl_3$ solution consisted of two units of OA.

In addition, an ESI mass spectrum performed on the natural isolated AOC, dissolved in methanol/water (85:15), exhibited peaks at *m*/*z* 1665 $[(OA)_2 + K + H_2O]^+$ and 1647 $[(OA)_2 + K]^+$. These data pointed out that the OAC studied by NMR consists of two units of OA and one potassium cation, proving at the same time that the dimer exists in aqueous solution. HR-DOSY experiments in water showed diffusion coefficients of $(2.82 \pm 0.02) \times 10^{-6}$ and $(2.69 \pm 0.05) \times 10^{-6}$ cm^2 s^{-1} for OA and OAC, respectively. This little difference suggests a monomer−dimer equilibrium in water, clearly shifted to the monomer species, according to the following expression:

$$D_{obs} = f_d D_d + (1 - f_d) D_m$$

where D_{obs} is the observed diffusion coefficient, f_d is the fraction of dimer, and D_d and D_m are the diffusion coefficients of the dimer and the monomer, respectively.

Once the presence of the OAC dimeric nature was established, we conducted a conformational study on the basis of the differences observed in ROESY spectra obtained for OA and OAC (Figure 4). In the ROESY spectrum of OAC two new signals appeared, correlating the following pairs of protons H-4/H-41 and H-12/H-26, and a significant increment in the intensity of the H-15/H-16 correlation signal was observed. The intensity change in the signal that correlates H-15 and H-16 could be explained by a rotation around the C15−C16 bond, but the other two nuclear Overhauser effects are consistent with our previous observations about the OAC dimeric nature.

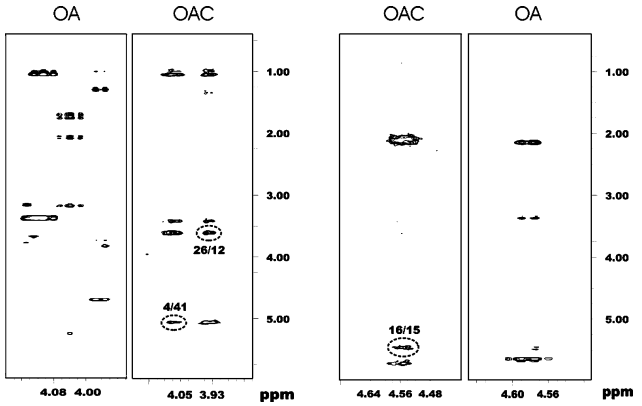

Figure 4. Selected strips extracted from 2D-ROESY spectra of OA and OAC. The new signals correlating H-4 with H-41 and H-12 with H-26 are marked. The significant change in the intensity of the H-15/H-16 cross-peak is also highlighted.

It is also important to note that duplication of signals is not observed in either the ^{1}H or the ^{13}C spectra for OAC, suggesting that all nuclei have equivalent magnetic environments and therefore are degenerate in chemical shift. This would cause "symmetry degeneracy", and only one monomer could be "seen".

On the basis of the lowest energy conformer of OA, obtained by Monte Carlo conformational search,[17] and by use of the data mentioned above, a symmetric dimer was built. The potassium cation was positioned into the cavity formed by two OA units, considering that the most notable differences between the ^{1}H spectra of OA and OAC correspond to the signals of the double bond H-14/H-15, the methines H-12 and H-23, and the methyl group Me-42 (Figures 2 and 5).

The optimal model was obtained following two steps. First, a constrained minimization, adjusting distance restrictions in accord with ROESY measurements, to the lower limit was done.[18] Here, the system was forced to be packed as much as possible,[19] in order to get the best interactions between the potassium cation and the oxygen atoms on both OA molecules.

In the second step, the system was allowed to relax using distance restrictions in which upper distance limits were adjusted to those obtained from NMR data (Tables 1 and 2).

According to the previous results, the potassium cation binding site is formed by three neighboring oxygen atoms attached to carbons C-7 and C-8 of each OA unit (Figure 6). Thus, the coordination proposed for the cation points out a trigonal bipyramid binding geometry involving two oxygens at spiro carbon C-8 and the oxygen present at C-7, which explains the spectroscopical differences between both species.

Although there are clear structural similarities between OA and the group of polyether antibiotics,[20] some differences are observed. In OA, no tetrahydrofuran rings are involved in the complexation, which is a general aspect of the antibiotics. Instead, two tetrahydropyran rings and a secondary hydroxyl group partici-

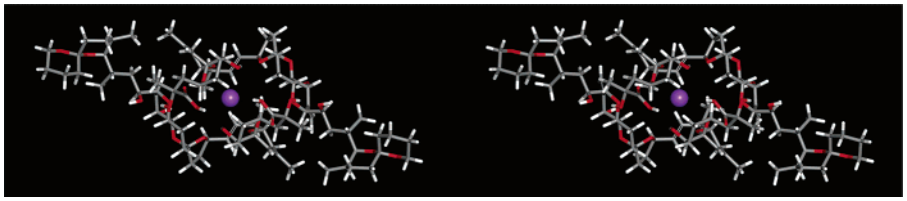

Figure 5. Stereoview (crossed eyes) of the proposed OAC structure. K^+ is shown as a purple sphere that is 0.3 times its van der Waals radius.

Table 1. Reference Intramolecular Distances Used To Calibrate the 2D-ROESY Spectrum

H′	H″	δ′ (ppm)	δ″ (ppm)	distance (Å)
4	5α	3.97	1.28	2.41
7	9	3.39	5.23	3.01
22	24	3.68	4.10	2.48
30	33β	3.30	1.59	3.84
33α	Me39	1.35	0.79	5.02
37α	38α	1.46	3.50	2.55

Table 2. Selected Intermolecular Distances

Ha[a]	Hb[a]	δa[a] (ppm)	δb[a] (ppm)	MDA[b] (Å)	distance[c] (Å)
4	41	3.98	5.12	2.0	4.83
5	26	1.29	4.00	2.0	7.59
12	13	3.64	2.26	3.6	8.72
12	26	3.64	4.00	2.0	2.96
12	30	3.64	3.29	4.6	7.82
13	12	2.26	3.64	3.6	8.72
13	28	2.28	0.93	4.0	7.29
24	Me42	4.10	0.94	4.4	5.70
26	5	4.00	1.29	2.0	7.60
26	12	4.00	3.64	2.0	2.97
28	13	0.93	2.28	4.0	8.44
30	12	3.29	3.64	4.6	7.98
41	4	5.12	3.98	2.0	4.85
Me43	Me39	1.69	0.79	2.0	3.74
Me42	24	0.94	4.10	4.4	5.64
Me39	Me43	0.79	1.69	2.0	4.24

[a] a and b refer to different okadaic acid monomers. [b] Maximum distance allowed in the first minimization step. [c] Distances calculated form the final model.

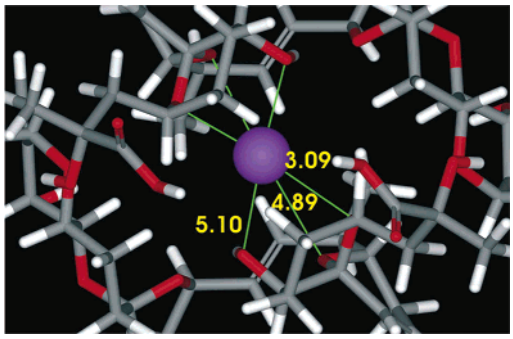

Figure 6. Proposed potassium ion coordination is shown by green lines. Selected distances (Å) between the ion and oxygen atoms are shown in yellow.

pate in the coordination of the potassium ion by OA. The carboxylate group of OA is not coordinated with the metal as occurs only in lenoremycin and monensin. The OA dimerization upon metal complexation is an unusual feature only shared by lasalocid and A23187.

In summary, structure determination of the OA-K^+ complex was carried out in $CDCl_3$, providing new insights into structural aspects of metal ion complexation by OA. We propose a pseudomacrocyclic structure stabilized by metal−oxygen electrostatic interactions and by inter- and intramolecular hydrogen bonds, which provides a rationalization, from a structural point of view, for the physiological importance of the potassium ion to the activity of OA. This structure, which has a hydrophilic coordination site and a more hydrophobic surface exposed to the solvent, may be a means of improving the membrane permeability of OA, in accordance with the reported K^+ dependence of OA activity in tissues.[6] The existence of a small fraction of the dimeric species in aqueous solution is in accord with the higher hydrophobicity of the complex, which forms more readily under hydrophobic conditions ($CDCl_3$, for instance, which may be regarded as a mimic of the interior of a lipid bilayer). Further studies with more biologically relevant membrane mimetics are in progress and will be reported.

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Supporting Information Available: The 2D NOESY and ROESY spectra for both AO and AOC in $CDCl_3$ and figures showing an OAC lipophilic potential surface and OA and OAC overlaid structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) The diffusion coefficients (*D*) are obtained from the calculation of the slope: γ^2, gyromagnetic ratio (rad s/g); *g*, strength of the gradients (G/cm); δ, length of the diffusion gradients (s); Δ, delay between the two diffusion pulse gradients (s).
(17) All calculations were done using SYBYL 6.7.1 (Tripos Inc., 1699 South Hanley Rd, St. Louis Missouri, 63144). Constrained minimization was done using Gasteiger−Hükel charges, $\epsilon = 80$, and 8 Å of dielectric distance cutoff. Tripos FF was used and minimization ended when the gradient was less than 0.05 kcal/(mol·A).
(18) ROESY analyses were done using the TRIAD module of SYBYL. Initial distance constraints (upper and lower limits) used were the defaults obtained from the software after calibration.
(19) This procedure has been shown to be useful for getting good contacts between molecules as a preparation of the system prior to doing further calculations (Tieleman, P. Theoretical Studies of Membrane Models. Ph.D. Thesis, University of Groningen, Groningen, The Netherlands, 1998; p 19).
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