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19-*epi*-Okadaic Acid, a Novel Protein Phosphatase Inhibitor with Enhanced Selectivity

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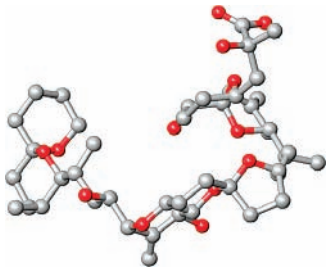
ABSTRACT



A new protein phosphatase inhibitor, 19-*epi*-okadaic acid, was isolated from the marine dinoflagellate *Prorocentrum belizeanum*. Its structure and conformation in solution has been determined, and important differences were found when compared with the lead compound okadaic acid. The new metabolite showed nanomolar activities, and its selectivity for PP2A versus PP1 surpasses that shown by okadaic acid 10-fold, making it one of the most selective inhibitors of this class.

Okadaic acid (OA), a polyether produced by dinoflagellates of the genus *Prorocentrum* and *Dinophysis*, has had an extraordinary impact upon different life science areas such as human health, seafood control analysis, pharmacology, natural product chemistry, fishery industry economics, etc., promoting new developments in all these areas.[1]

Originally isolated from a marine sponge as a result of a screening program undertaken by a pharmaceutical company in search of new cytotoxic compounds,[2] OA was subsequently characterized as the main agent responsible for diarrethic shellfish poisoning (DSP),[3] as well as being a potent tumor promoter.[4] The latter discovery was a decisive clue to unlocking the secret of its mechanism of action,[5] which involves selective inhibition of serine/threonine protein phosphatases (PPs).[6] Therefore, OA is now recognized as the first member of the "okadaic acid class" of PPs inhibitors, a remarkably different panel of metabolites that have become valuable tools for studying the cellular roles of different PPs.[7] In fact, the recognition that OA was an inhibitor of PPs played a decisive role in bringing these enzymes to the forefront of cellular and biochemical research. As a consequence, the essential role of PPs in the regulation of processes such as metabolism, signal transduction, cell growth, and control of programmed cell death, etc. has been accepted. Nowadays, OA is used in studies directed toward understanding diseases such as cancer, Alzheimer, diabetes, inflammation, etc. Not surprisingly, these findings have prompted a number of laboratories to pursue the total

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synthesis of the compound.[8] While structural studies of PP1 and PP2A are under active investigation, the finding of new inhibitors and the understanding of their conformational properties in solution has the potential to provide valuable insights into the binding processes.[9]

In the present study, we report on the isolation, structure determination, and inhibitory potency of a new okadaic acid analogue, 19-*epi*-okadaic acid (19-*epi*-OA), which showed important differences in its three-dimensional structure as well as in its pharmacological activity.

A unialgal strain large-scale culture of *P. belizeanum* (1000 L) was carried out for 45 days using Guillard K medium at 23 ± 1 °C under 16 light: 8 dark cycle in 80 L tanks. Afterward, cells were harvested by centrifugation at 3700*g*, immediately sonicated, and exhaustively extracted with acetone. Solvent was evaporated in vacuo obtaining a yellow-brown oil (22 g) that was successively fractionated using Sephadex LH-20, LiChroprep RP18, and HPLC equipped with an XTerra column, yielding 70 mg of purified OA together with 1 mg of the new toxin 19-*epi*-OA.

The molecular formula for 19-*epi*-OA was determined to be $C_{44}H_{68}O_{13}$ on the basis of the mass spectrum that presented an ion at *m*/*z* 827.4576 corresponding to $[C_{44}H_{68}O_{13} + Na]^+$, identical to OA.

Analysis of the NMR data[10] (Table 1) revealed the presence of five methyl groups, 16 methylenes (one sp^2), and 16 methines (three sp^2). Among them, one methylene and eight methines were ascribed to those bearing oxygen atoms, as was deduced from the analysis of the HSQC. The remaining six carbon atoms were quaternary, and their presence in the molecule conveniently divided it into five separated proton−proton coupled spin systems. Extensive analyses of the $^1H-^1H$ COSY and TOCSY spectra revealed connectivities which define five partial structures: I (H3−H7), II (H11−H18), III (H20−H24), IV (H26−H33), and V (H35−H38). Connections among the above fragments were established using $^1H-^{13}C$ long distance correlations extracted from the HMBC experiment (Figure 1).

Table 1. ^1H and ^{13}C NMR Spectral Differences Observed in CD_3OD for 19-*epi*-Okadaic Acid versus Okadaic Acid

	19-*epi*-okadaic acid		okadaic acid	
C	δ ^{13}C	δ ^1H	δ ^{13}C	δ ^1H
15	131.1	5.56	131.9	5.34
16	82.9	4.43	80.2	4.52
17	31.6	1.98/1.81	31.0	2.08/1.43
18	33.3	2.36/1.57	37.7	1.88/1.72
19	109.0		106.5	
20	34.4	1.82/1.74	33.4	1.84/1.72
21	28.7	1.92/1.45	27.5	1.90 /1.75
22	70.9	3.60	71.1	3.50
23	81.8	2.93	78.1	3.28
24	71.3	4.05	71.8	3.92

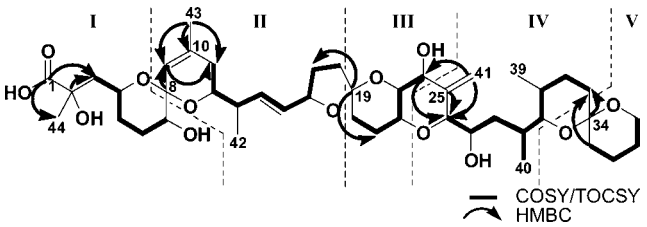

Figure 1. NMR-derived correlations observed for 19-*epi*-okadaic acid.

On the basis of the above analysis, the planar structure of 19-*epi*-OA was assigned as identical to OA, although significant differences were found in the ^1H and/or ^{13}C chemical shifts of the fragment C15−C24 (Table 1).

Therefore, we concluded that the observed spectroscopic discrepancies must arise from differences in the configuration of at least one chiral center located at the previously mentioned fragment of the new structure.

A qualitative analysis of the ROESY experiment focusing on this fragment showed enough dipolar correlations to establish the stereochemistry of all the chiral centers present in the molecule. Thus, the ROESY of the new compound showed significant cross-correlations, which allowed us to determine the configurations of C22, C24, C26, and C27 as identical to those present in OA. However, the new clear cross-correlation between H18 and H23 which was observed could only be explained if the new compound had C19 epimerized (Figure 2). Therefore, we determined the structure of the new compound to be the 19-epimer of OA.

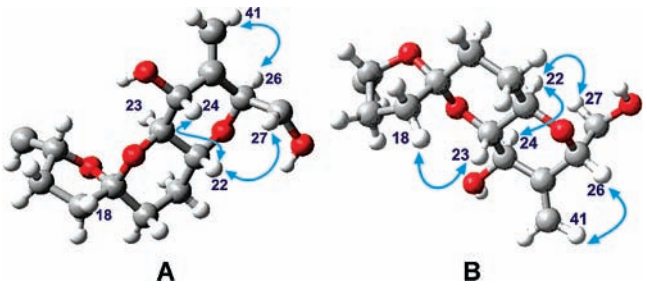

Figure 2. Relevant ROEs observed for okadaic acid (A) versus 19-*epi*-okadaic acid (B) indicated by arrows in their corresponding C15−C27 fragments.

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(10) NMR experiments were acquired in deuterated methanol at 400 and 500 MHz at a temperature of 298 K. HSQC, COSY, 1D/2D TOCSY, HMBC, and ROESY experiments were performed using standard pulse sequences. Data were processed using MestRec software. The volume integrals of the individually assigned NOE cross-peaks were converted into distance constrains using the isolated spin pair approximation, taking into account the offset effect.

The conformation of 19-*epi*-OA in CD_3OD solution was determined using 39 interproton distances, extracted from ROESY experiments, and seven $^3J_{HH}$ coupling constants, measured from phase-sensitive DQF-COSY, as restrains in MonteCarlo conformational searches. Three different ROESY spectra were obtained using mixing times of 200, 300, and 500 ms. Volume integrals of the individually assigned cross-peaks were converted into distance restrains taking into account the offset effect, using a distance of 2.57 Å between H30 and H32^{proR} as an internal calibration.[11]

The crystal structure of OA was used as a starting point to build the structure of 19-*epi*-OA by inverting the configuration of C19.[12] The latter structure was used as the starting point for conformational searches. These consisted of four separate searches with the MM3*[13] and MMFF94s[14] force fields as implemented in MacroModel[15] 8.5 either using a bulk dielectric constant of 32.7 (equivalent to the dielectric constant of methanol) when calculations were performed in vacuo or combined with the generalized Born/surface area (GBSA) water solvent model.[16] Each force field was applied to separated random-seeded searches of 20 000 MCMM (MonteCarlo Multiple Minimum) steps to ensure that the potential energy surface was thoroughly explored using the TNCG algorithm. All local minima within 50 kJ of the global minimum were saved and re-minimized with their respective force fields using the FMNR algorithm and an energy cutoff of 25 kJ.[17] The ensemble of the 10 lowest energy structures is consistent with the observed ROEs showing only three minor ROE violations (<0.20 Å) and a rmsd of 0.60 Å after alignment (Figure 3).

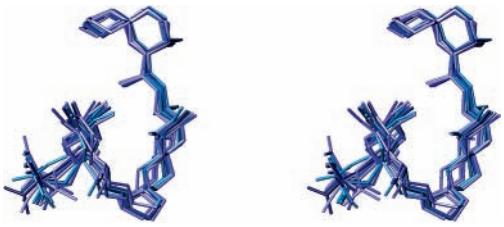

Figure 3. Stereoview of the 10 best NMR-derived conformers obtained for 19-*epi*-okadaic acid.

Despite the potential flexibility of this molecule, the conformational searches provided a convergent array of structures. Evident in all minima was a major turn in the carbon backbone inducing a left-hand pseudo-helical twist to the scaffold. Unrestrained molecular dynamics (MD) were run at 300 K for 2.5 ns in order to validate the results obtained from the conformational searches.

According to the MD simulations, the previously obtained conformation is moderately flexible along the backbone with higher variation for the C1−C4 fragment. This result can be explained if we consider that a key difference of 19-*epi*-OA with respect to OA is that the carboxylic acid (C1) and the hydroxyl group located at C24 are now situated on different faces of the molecule, hindering the possibility to form a hydrogen bond between them (Figure 4). The

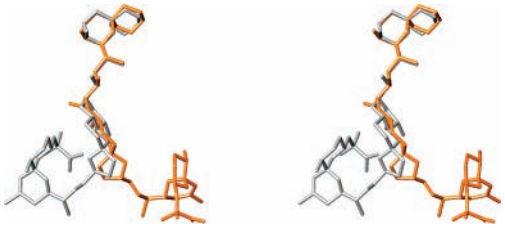

Figure 4. Stereoview of the NMR-derived conformation for 19-*epi*-okadaic acid (orange) superimposed on the X-ray structure of okadaic acid (gray).

presence of such a hydrogen bond has been mentioned several times as the key factor which determines the stabilization of a pseudo-macrocyclic structure in OA.[18] However, in this new compound, we have found that this is not necessarily the case. The absence of this intramolecular hydrogen bond makes 19-*epi*-OA more polar than OA, as demonstrated by the fact that it is soluble in methanol but not in chloroform.

A detailed conformational analysis of the potential hinge region of the molecule showed that, in the C12−C16 moiety, the average calculated dihedral angles between H12−H13, H13−H14, and H15−H16 were characteristic of *anti* orientations. Only small variations in these angles were observed during the MD simulations; however, these movements still confer some flexibility to the molecule as can be observed from the variation of the distance between the oxygen atoms located at C7 and C24, which fluctuates around a mean value of 5.25 ± 0.92 Å during the MM3* MD trajectory. Finally, no major changes were observed in the C26−C30 region, which is characterized by dihedral angle values well-defined around 180° between the protons H26−H27, H27−H28^{proR}, H28^{proS}−H29, and H29−H30. MD simulations using different conditions starting from different local minima gave rather similar and stable trajectories indicating the conformational stability of these geometries.

The biological activity of the new compound was assessed using the two main targets of OA, the serine−threonine

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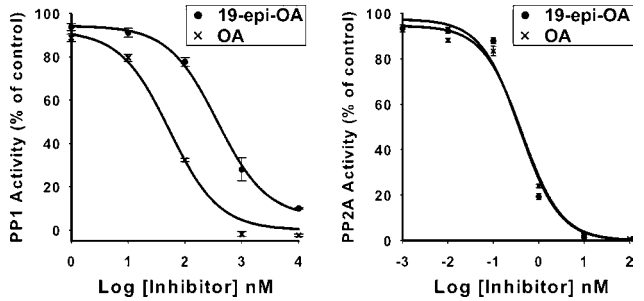

Figure 5. Inhibition of protein phosphatases type 1 and 2A by 19-*epi*-okadaic acid and okadaic acid.

protein phosphatases type 1 and 2A (Figure 5).[19−21] OA is the archetypical inhibitor of both enzymes and was therefore used as a control. 19-*epi*-OA potently inhibited PP2A, showing an IC_{50} value of 0.47 ± 0.04 nM, virtually equipotent to OA which showed an IC_{50} of 0.58 ± 0.05 nM. However, the inhibitory activity of the toxins versus PP1 showed a different scenario as 19-*epi*-OA presents an IC_{50} of 465 ± 52 nM while OA inhibits such enzyme more potently, with an IC_{50} of 62 ± 6 nM.

Most toxins that inhibit PP1 and PP2A share two characteristics: a carboxyl group and a hydrophobic tail;[22] in addition many include a large macrocyclic domain fixing a conformation that allows the inhibitor to lock into at least two of three adjacent groves located at the active site of protein.[23] All these structural features were observed in the X-ray structures of OA, either free or in complex with the proteins.[11,24,25] In both situations, OA showed a very similar conformation, including the hydrogen bond between the C24 hydroxyl and the C1 carboxylic acid. On the other hand, the comparison of the conformations in solution of OA versus 19-*epi*-OA showed a major difference: while OA shows a right-hand twist, 19-*epi*-OA turns in the opposite direction, therefore preventing the formation of the C24/C1 hydrogen bond. As a result, the contact points between the new inhibitor and the protein should be different than those found in the OA−PP1/PP2A complexes. This is something that makes interpretation of the relative inhibitory potencies of 19-*epi*-OA in structural terms difficult, especially in the case of PP2A, where both inhibitors are equipotent. At this point, it has to be considered that the absence of the C24/C1 hydrogen bond confers a higher flexibility to 19-*epi*-OA, which could be of great importance as it may provide this molecule the necessary plasticity to fit into the active site of the protein. Finally, it should be noted that 19-*epi*-OA is a protein phosphatase inhibitor with PP2A/PP1 specificity 10-fold better than OA, making it one of the most selective PP2A inhibitors. This is also an important point since from the analysis of protein−ligand interactions significant conclusions about the structural motifs necessary to design specific PP1 or PP2A inhibitors could be extracted. Further structural analysis of the complexes formed by PPs and 19-*epi*-OA is currently in progress and will be reported in due course.

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Supporting Information Available: NMR data and results from conformational searches and MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) PP1c was purchased from New England Biolabs, and PP2Ac was purchased from Sigma. Protein phosphatase activity was assayed at 37 °C using 0.025 units/μL of protein and 100 μM of fluorescein diphosphate as substrate. The reaction was followed by fluorescence spectroscopy at 485−535 nM. Data were fitted to a one-site binding model. Assays with 19-*epi*-okadaic acid were performed in quintuplicate ($n = 5$), while for okadaic acid only duplicates ($n = 2$) were used.

(21) Assays adding BSA (0.125 mg/mL) to the media were also undertaken in order to explore the possibility that 19-*epi*-OA could be a "phony" hit as described in McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. *J. Med. Chem.* **2002**, *45*, 1712−1722, but no significant differences were seen, leaving out such possibility.

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