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Corozalic Acid: A Key Okadaic Acid Biosynthetic Precursor with Phosphatase Inhibition Activity

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Dedicated to Professor José Barluenga on the occassion of his 70th birthday

Protein phosphatases (PPs) play an essential role in the regulation of key cellular processes such as metabolism, signal transduction, cell growth and apoptosis.[1] Within this family of enzymes, serine/threonine protein phosphatases[2] have recently emerged as important targets for chemical biology and medicinal chemistry research because of their clear potential to become drug targets for the treatment of diseases such as cancer, diabetes, Alzheimer.[3] However, although significant achievements have been made, the development of selective PPs inhibitors is still in its early stages.[4]

Okadaic acid (**1**), one of the major toxins associated with *Prorocentrum spp*. and diarrheic shellfish poisoning (DSP) syndrome,[5] was also the first marine natural product described to selectively and potently inhibit protein phosphatases type 1 (PP1) and type 2A (PP2A).[6] Therefore, **1** has proved to be an indispensable tool in physiological and pharmacological studies, enhancing our understanding of specific function and regulation of Ser/Thr protein phosphatases.[7] Although the majority of naturally occurring analogues of **1** possess only slight structural modifications on its main carbon backbone, the recent isolation of new inhibitors belonging to the "okadaic acid class" has opened new possibilities for the development of useful modulators of the catalytic activity of protein phosphatases.[8]

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In the present study, we describe the isolation, structural elucidation and biological activity of corozalic acid (**2**), a new serine/threonine phosphatase inhibitor structurally related to okadaic acid, obtained from cultures of *Prorocentrum belizeanum*. In addition, we present a plausible biosynthetic pathway where both compounds originate from a common linear precursor.

A large-scale culture of the dinoflagellate *P. belizeanum* (strain PBMA01) was incubated statically for 50 days at 23 °C using 750 L of seawater enriched with Guillard-K medium.[9] Cells were harvested by centrifugation at 3700 × *g*, extracted with acetone (5 × 800 mL) and then re-extracted with methanol (5 × 800 mL). The methanolic extract (14.95 *g*) was purified by chromatography on a Sephadex LH-20 column (CH_3OH) and then fractionated using a Lobar LiChroprep RP8 column eluted with CH_3OH/H_2O (7:3). Subsequent gel-filtration chromatography on a Sephadex LH-20 column eluted with $CH_3OH/CHCl_3$ (1:2) proved to be essential to separate minor constituents from okadaic acid (35:1 ratio). Final purification of **2** was achieved by semipreparative reversed-phase HPLC (XTerra Prep C18 10 μm, 1.0 mL min^{-1}) using an isocratic elution of CH_3OH/H_2O (17:3), thus obtaining 0.6 mg of the new compound.

Corozalic acid was obtained as an optically active, white amorphous solid ($[\alpha]_D^{25} = +5.1$ ($c = 0.08$, CH_3OH)). HR EIMS analysis of **2** showed a molecular ion peak at *m*/*z* 822.4762, thus establishing the molecular formula $C_{44}H_{70}O_{14}$, with a total of 10 degrees of unsaturation. The ^{1}H NMR spectrum of **2** clearly resembles that of **1**, and the main differences are the downfield shift of the proton H-15 and the upfield shift observed for protons H-14 and H-12 (Table 1).

Heteronuclear 2D NMR spectroscopy showed that **2** contains sixteen methines, sixteen methylenes, five methyl groups, and seven quaternary carbons including one carboxylic acid functionality (δ_C 178.9 ppm), consistent with the absorption band observed at $\tilde{\nu} = 1727$ cm^{-1} in the IR spectrum. The resonances located at $\delta_C = 123.5$, 132.8, 137.0

Table 1. ^{13}C and ^{1}H chemical-shift differences (in ppm) observed in CD_3OD for okadaic acid versus corozalic acid.

	Okadaic acid (**1**)		Corozalic acid (**2**)			
Carbon	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	$\Delta\delta$ ^{13}C[a]	$\Delta\delta$ ^{1}H[b]
7	73.2	3.22	73.3	3.26	+0.1	+0.04
8	97.1		97.9		+0.8	
9	123.5	5.13	123.7	5.18	+0.2	+0.05
10	138.8		139.9		+1.1	
11	33.1	1.90	34.3	1.82	+1.2	−0.08
		1.69		1.82		+0.13
12	71.4	3.71	72.1	3.58	+0.7	−0.13
13	42.9	2.20	43.4	2.25	+0.5	+0.05
14	137.2	5.81	137.0	5.71	−0.2	−0.10
15	131.9	5.34	132.8	5.48	+0.9	+0.14
16	80.2	4.52	80.7	4.46	+0.5	−0.06

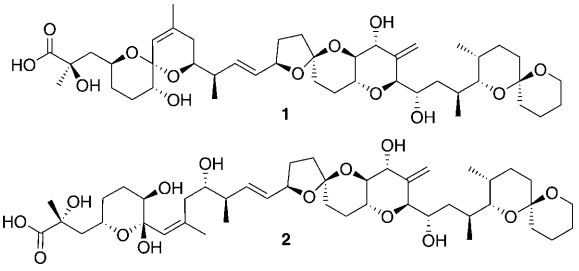
[a] $\Delta\delta$ $^{13}C = \delta$ ^{13}C(**2**) $-\delta$ ^{13}C(**1**). [b] $\Delta\delta$ $^{1}H = \delta$ ^{1}H(**2**) $-\delta$ ^{1}H(**1**).

and 139.9 ppm indicated the presence of two olefins, while the signals at δ_C = 112.6 (methylene) and 147.3 ppm (quaternary carbon) established the presence of an *exo*-type double bond. Taken together, these data account for four out of the ten degrees of unsaturation indicated by the molecular formula, thus suggesting that **2** contains six rings on its structure instead of the seven rings that are present in **1**.

In a similar way to what it is observed in **1**, 2D COSY and TOCSY experiments showed the presence of five discrete ^{1}H–^{1}H spin systems in **2**: **I** (H-3/H-7), **II** (H-11/H-18), **III** (H-20/H-24), **IV** (H-26/H-33) and **V** (H-35/H-38). This time, in particular, all changes were assigned to fragment II.

Analysis of selective 1D TOCSY and 2D COSY experiments revealed correlations between H-14 (δ_H 5.71) and protons H-15 (δ_H 5.48) and H-13 (δ_H 2.25). The latter proton was then correlated to oxymethine H-12 (δ_H 3.58) and to methyl group H_3-42 (δ_H 0.96). In the same way, ^{1}H–^{1}H correlations were identified between H-12 and methylene H_2-11 (δ_H 1.82), as well as between H-15 and oxymethine H-16 (δ_H 4.46). Analysis for this particular spin system (II) was completed when H-16 was connected to diastereotopic protons H_2-17 (δ_H 2.11/1.50) and H_2-18 (δ_H 1.92/1.75). The previous situation clearly resembles that observed for **1**, but now the molecular formula indicates the existence of an additional hydroxyl group as well as one unsaturation less. These data together with the chemical shift changes observed in the C-11/C-15 region led us to the conclusion that the structure of **2** is that shown below.

The relative stereochemistry of C-8 was assigned as *S** according to the clear ROE connectivity observed between H-7 (δ_H 3.26) and H-9 (δ_H 5.18). On the other hand, a different



approach (*J*-based configurational analysis) was used to elucidate the relative configuration of C-12, as this chiral center is located in the middle of an acyclic chain.[10] $^{3}J_{H,H}$ and $^{2,3}J_{C,H}$ values were obtained from ^{1}H NMR, DQF-COSY and HSQC-HECADE[11] spectra. The average value found for $^{3}J_{H\text{-}12,H\text{-}13}$ (7.3 Hz) indicates a conformational interconversion around the C-12/C-13 bond. From the small values found for $^{3}J_{C\text{-}11,H\text{-}13}$ (2.6 Hz) and $^{3}J_{C\text{-}42,H\text{-}12}$ (1.4 Hz), a pair of alternating rotamers with *erythro* configuration was assigned, thus indicating that the relative configuration of C-12 was *S** (Figure 1). The relative stereochemistry for the rest of the chiral centers in **2** was confirmed to be identical to that of **1**, according to ROESY experiments in combination with coupling constants measurements. A conformational search was also undertaken and the results were consistent with the experimental data (see Supporting Information).

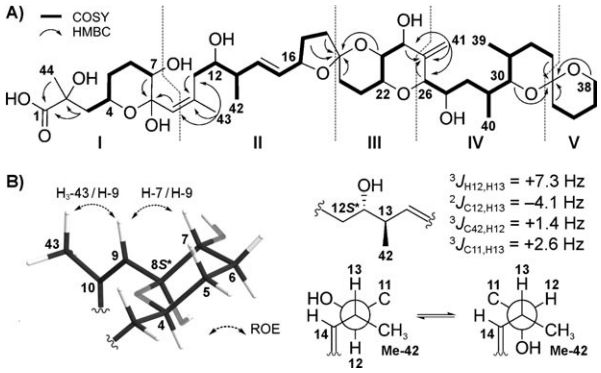
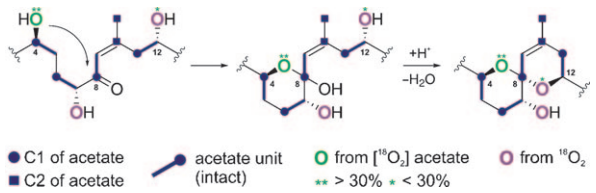


Figure 1. A) COSY/TOCSY fragments and selected HMBC correlations for corozalic acid. B) Stereochemical assignment of C-8 and C-12 in corozalic acid.

The isolation of **2** is quite important from a biosynthetic point of view. Even though the origin of all atoms present in **1** have been established on the basis of feeding experiments using isotopically enriched precursors and that based on those results Murata et al.[12] proposed a mechanism to explain the formation of the tricyclic polyether system of **1**, the chance of confirming that hypothesis has been severely hindered by the inability of the dinoflagellate to incorporate more elaborate precursors. In fact, the best support for such proposal has been the isolation of belizeanic acid.[8a]

Considering the structural similarities between **2** and **1**, it is reasonable to propose a common biogenetic pathway for both metabolites, where **2** is an intermediate in the biogenesis of **1**. Using a mechanism similar to that proposed for the spiroketal moieties found in the avermectins/milbemycins family of antibiotics,[13] ring A in **2** would be formed from a linear precursor through a nucleophilic attack of the C-4 hydroxyl group to the carbonyl group at C-8, yielding the hemiketal moiety. Next, a nucleophilic attack of the hydroxyl group at C-12 onto the hemiketal at C-8 or from the hydroxyl at C-8 to C-12, as part of a S_N1 reaction, would generate the characteristic [6,6]-spiroketal ring system of **1**.

The proposed mechanism is supported by the fact that the oxygen bridge between C-8 and C-12 derives from both acetate and O_2 (Scheme 1),[12] and is consistent with deuterium



Scheme 1. Biosynthetic proposal for the C-4/C-12 fragment of corozalic acid (**2**) and okadaic acid (**1**).

retention at C-5, C-9 and C-12.[14] Finally, the fact that the relative stereochemistry of C-4 and C-12 in **1** is identical to that observed in **2** can be explained by both nucleophilic attacks, as the "best-energy" structures found in the conformational search seem to be preorganized in a way that would give the correct stereochemistry (see Supporting Information). In addition, an observation that substantiates our hypothesis is that **2**, although very slowly, transforms into **1** when it is stored in solution. This observation could be also interpreted as indicative of the existence of a reversible conversion of **1** into **2**. In our opinion, this possibility should be ruled out, as we have never observed the transformation of **1** into **2** even tough we have stored several samples of the former for years under similar conditions. To be precise, we have examined samples of **1** kept in water, methanol or chloroform. In addition, other authors who have undertaken structural studies of **1** under basic conditions did not report the transformation of **1** into **2**.[15] Finally, it has to be noted that several analytical studies directed to address the DSP problem using different extraction and purification protocols have been performed by LC/MS without noticing the conversion of **1** into **2**.[16]

Corozalic acid was evaluated for in vitro activity as PP1 and PP2A inhibitor and IC_{50} values of 584.2 ± 26.1 and 53.3 ± 2.3 nM, respectively, were found. These figures were compared with those obtained by us for **1** (73.6 ± 4.2 with PP1 and 2.2 ± 0.8 nM with PP2A). The results denote that **2**, although in smaller relative proportion, still has higher affinity for PP2A than for PP1. This fact has been explained for **1** by the existence of a "hydrophobic cage" inside of the binding site of PP2A, that is not conserved in PP1, which surrounds the common C-30/C-38 hydrophobic moiety. At this point it is important to remember that all the structural differences between **1** and **2** are located in the C-7/C-12 segment and that in the crystallographic structures of **1** in complex with these enzymes, this part of the ligand interacts with the β12–β13 loop of both proteins. However, our data indicate greater reduction of the binding affinity of **2** for PP2A in comparison with PP1 (twenty-five- and eight-fold less, respectively).

In an attempt to understand the basis of the observed differences in activity, computational simulations were performed. PP1 and PP2A are metal-dependent enzymes, thus due to the existence of two Mn^{2+} ions in the active site we calculated the atomic charges of all residues within a 3 Å distance of the inhibitor (including a water molecule coordinated to both manganese ions) using ab initio calculations at the B3LYP/6-31G** theoretical level. Next, the inhibitor was docked into the active site of the protein using the AutoDock software.[17] Based on the observation that in all the crystallographic structures available, the distance between the carboxylic acid and the manganese ions is larger than 4.5 Å, docking solutions where such requirement was no fulfilled were discarded.[18] Finally, molecular dynamics (MD) simulations using an explicit water model were undertaken in order to refine the previous structures.[19] Since the active binding site of these phosphatases are almost identical in all the crystal structures that have been determined, harmonic restraints were applied to the enzyme backbone.[20,21] As a first control, the structure of the complexes formed by **1** and PP1 and PP2A were calculated using the corresponding crystallographic coordinates of the protein (pdb 1JK7 and 2IE4),[22,23] obtaining excellent results compared to the experimental data (RMSD 0.40 and 0.47 Å, respectively). Then, as an additional control, the binding mode of calyculin, a structurally different inhibitor, was predicted using the previous protocol and compared with the crystallographic structure (pdb 1IT6)[24] obtaining high-quality results as well (RMSD 0.5 Å). Finally, in order to identify major contributions to binding, MD outputs were evaluated using DrugScore.[25] (see Supporting Information).

According to our results (Figure 2), the binding mode of **2** to PP1 and PP2A involves several favorable contacts previously identified in the corresponding crystal structures of **1** in complex with both proteins. These include key hydrogen bonds of the α-hydroxy carboxylic moiety and the hydroxy group located at C-24 as well as important hydrophobic interactions of the C-25 *exo*-type double bond and the C-30/C-38 spiroketal moiety. On the other hand, differences emerge near the β12–β13 loop. Thus, analysis of the results obtained for the PP2A–corozalic acid complex suggested that the overall loss of activity compared to okadaic acid (twenty-five-fold) is mainly a consequence of the negative balance between the favorable interaction involving the new hydroxyl group at C-12 and Cys269 contrary to the adverse contacts of its vicinal methyl group C-42 with Leu243, together with the absence of favorable interactions of **2** with residues Cys266 and Arg268 observed in the complex with **1**. However, in PP1 Phe276 occupies the position of Cys269 in the β12–β13 loop, resulting in a smaller binding pocket and as a consequence the C-8/C-16 acyclic portion of **2** moves outside of it. Accordingly, the hydrophobic interaction of the methyl group C-42 of **2** with Val250 observed in the PP1–**1** complex is now absent as well as contacts between methyl C-43 and Cys273, Glu275 and particularly with Tyr272 that are less favourable than those observed for **1**. On the other hand interactions with Phe276 seem to be better for **2** than for **1**.

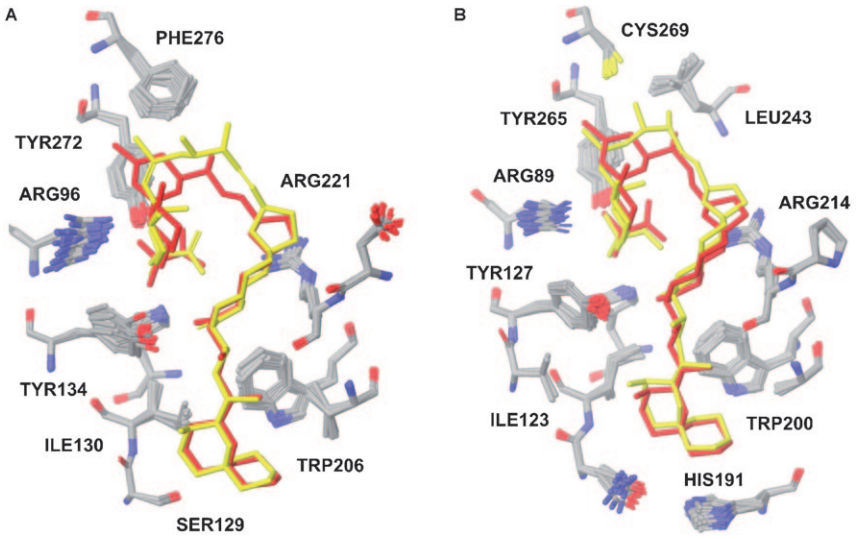


Figure 2. Comparison of the crystal structure of okadaic acid (**1**, red) and the representative structure of corozalic acid (**2**, yellow) calculated from the last 300 ps of the MD trajectory. Snapshots of key residues within the active site during the MD simulation are shown. A) PP1. B) PP2A.

Assuming the IC_{50} values can be roughly matched with the K_i values,[26] then $\Delta\Delta G = -RT\ln[K_i(\mathbf{1})/K_i(\mathbf{2})]$ is 5.34 kJ mol^{-1} for PP1 and 8.22 kJ mol^{-1} for PP2A at 310 K. Considering that a value of 3.95 kJ mol^{-1} was calculated for the difference in torsional free energy loss upon binding between **1** and the more flexible ligand **2**,[17] the energetic differences left of 1.39 kJ mol^{-1} for PP1 and 4.27 for PP2A (favorable in both cases to **1**) should be explained by the slightly better interaction of okadaic acid with the β12–β13 loop in both proteins.

Finally, with regard to the identification of a refined pharmacophore model for PP1 and PP2A,[27] we believe that our results suggest that the existence in **2** of a new hydroxyl group at C-12 could increase the selectivity for PP2A (hydrogen bond with Cys269) but this trend is opposed by the occurrence of its vicinal methyl group C-42, which clashes with Leu243.

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Keywords: biosynthesis · enzyme inhibitors · molecular modeling · natural products · okadaic acid

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