Prorocentric Acid, a Neuroactive Super-Carbon-Chain Compound from the Dinoflagellate *Prorocentrum hoffmannianum*

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Supporting Information Placeholder

ABSTRACT: Prorocentric acid (PA) was isolated from the dinoflagellate *Prorocentrum hoffmannianum*. Relative configurations for its 35 asymmetric centers, were determined by analysis of NMR data including heteronuclear couplings and quantum mechanical calculations. PA was tested by using murine cortical neurons grown on microelectrode-arrays. Long term exposure to subtoxic concentrations induced a significant reorganization of neuronal signaling, mainly by changes in the bursting activity. The observed effects could be due to the activation of a plasticity process.

Marine dinoflagellates produce an unparalleled variety of complex metabolites with diverse potent bioactivities.^{1–3} In particular, the genus *Prorocentrum*, is well known for producing the diarrhetic toxin okadaic acid and several derivatives.^{4–7} Due to its exquisite specificity for Ser/Thr-protein phosphatases 1 and 2A it has played an important role in the study of their biochemistry and in pathologies such as Alzheimer's disease.^{8,9} *Prorocentrum* also produces bioactive macrolides, polycyclic and super-carbon chain compounds, such as hoffmannioid, ¹⁰ prorocentrolides, ¹¹ spiro-prorocentrimine, ¹² prorocentrol, ¹³ prorocentrin, ¹⁴ and belizentrin. ^{15,16} Novel super-carbon-chain structures such as belizeanolide and belizeanolic acid have also been isolated from the culture media.¹⁷

Figure 1. Molecular structure and relative configuration of prorocentric acid.

The culture medium of *P. hoffmannianum*, was extracted using a Diaion HP-20 column, to obtain 3.2 g of crude extract, which was chromatographed in a Lobar LiChroprep RP-8 column eluted with CH₃OH:H₂O (1:1). Selected fractions were further fractionated using Sephadex LH-20 eluted with CH₃OH and CHCl₃:CH₃OH (2:1) in two successive steps. Final purification was achieved using an RP-18 HPLC column, yielding 7.4 mg of prorocentric acid (PA) (Figure 1). MALDI-TOF mass spectra showed a pseudomolecular ion [M-H][−] at *m/z* 1400, while ESI-FTMS provided the molecular formula [MNa+H]⁺ C₆₅H₁₀₈O₃₂Na (calc. 1423.6716; obs. 1423.6711) and [MK+H]⁺ C₆₅H₁₀₈O₃₂K (calc. 1439.6455; obs. 1439.6450) corresponding to a compound with 12 degrees of unsaturation. In fact, 65 signals were observed in the ¹³C NMR spectra. The HSQC spectrum revealed the existence of 5 methyls, 13 methylene carbons, 9 protonated olefinic carbons and 33 methines (29 bearing oxygen). Comprehensive analysis of 2D NMR data allowed the assignment of four ¹H-¹H spin systems, that were connected using ¹H,¹³C long-range correlations extracted from the HMBC experiment (Figure 2). To overcome signal overlap, NMR data were acquired in methanol-*d*₄ as well as a 1:1 mixture of methanol-*d*₄:pyridine-*d*₅. This simple strategy was particularly useful to connect the C16-C18 and C28-C30 positions. Deuterium shift experiments using methanol-*d*₃ showed no changes for C6, C10, C17, C21, C29, C33, C34, C35 and C36 indicating that these carbons were attached to non-protonated oxygens.

Figure 2. Selected HMBC correlations observed in prorocentroidic acid.

Furthermore, two oxanes, one oxirane and one bicyclic ring were built based on the observation of $^3J_{C,H}$ long-range correlations between C6-H10 and C17-H21 together with those between C-29-H33 and C29-H34. Once the planar structure of PA was determined as shown in Figure 2, we explored the stereochemical features of this complex molecule. Hence, an *E* configuration was assigned to all double bonds based on the observed dipolar correlations and $^3J_{H,H}$ values obtained from selective 1D-TOCSY experiments. Particularly important for the Δ^{13-14} and Δ^{49-50} olefins were the observed ROEs between CH₃-65 and H15 as well as those between the pairs H48 and H50 and H49 and H51. $^3J_{H,H}$ values measured from selective 1D-TOCSY experiments (15.5, 15.0, 12.2 and 16.4 Hz for Δ^{3-4} , Δ^{41-42} , Δ^{49-50} and Δ^{56-57} , respectively) were also significant. Next, we continued studying all cyclic systems, as shown in Figure 3. Thus, configurations within the C6-C10 ring were determined by observation of ROEs between H6, H8 and H10, locating H7 and H9 on the other face. Large coupling constants $^3J_{H6,H7}=7.3$ Hz, $^3J_{H7,H8}=10.2$ Hz, $^3J_{H8,H9}=8.5$ Hz, $^3J_{H9,H10}=9.2$ Hz confirmed their antiperiplanar relationship and suggested a chair conformation with an equatorial orientation of substituents at C6 and C10 (Figure 3A). Relative configurations within the C17-C21 oxane ring were determined on the basis of the measured $^3J_{H,H}$ values ($^3J_{H18,H19}=4.2$ Hz, $^3J_{H19,H20\alpha}=5.2$ Hz, $^3J_{H19,H20\beta}=3.8$ Hz, $^3J_{H20\alpha,H21}=4.0$ Hz, $^3J_{H20\beta,H21}=11.8$ Hz) and ROESY data. (Figure 3B). The stereostructure of the C29-C34 bicycle was mostly unravelled by observation of a ROE network from H34 to H33, H31 and methyl C62 as shown in Figure 2. In addition, the $^3J_{H33,H34}=1.0$ Hz value on the five-membered ring was compatible with the selected configurations (Figure 3C).¹⁸

Figure 3. Selected NMR dipolar correlations (dashed lines) observed in prorocentroidic acid.

Relative configurations along the acyclic fragments were determined using a *J*-based coupling constant (JBC) analysis.¹⁹ Thus, a *threo* relative configuration along the C5-C6 bond was set on the basis of the measured small homonuclear constant ($J_{H5,H6}=1.2$ Hz) and long-distance heteronuclear constants ($J_{H6,C4}=1.5$ Hz, $J_{H6,C5}=1.5$ Hz, $J_{H5,C6}=1.4$ Hz). (Figure 4) gave a relative configuration of 5*S**,6*R**,7*S**,8*R**,9*R**,10*S**. The second stereocluster, C15-C25, included 9 asymmetric centres. A *threo* relationship along the C15-C16 single bond was assigned on the basis of $^3J_{H15,H16}=9.8$ Hz, characteristic of an *anti* relationship, and the observed ROE between H14 and H18 (Figures 3 and 4). For non-protonated positions, such as C-17, the reduced set of available NMR restraints prevented the use of JBC analyses. However, quantum mechanics (QM-NMR) methods can overcome this difficulty.²⁰ In fact, they are gradually becoming a quicker and sometimes even more reliable alternative than total chemical synthesis.^{21,22} Among them, methods based on the so-called DP4 probability stands out.²³ Thus, truncated models were built and calculations for the four possible diastereoisomers were performed to predict the 15*R**, 16*S**, 17*S**, 18*S**, 19*S**, 21*R**, 22*R**, 23*S**, 25*R** stereoisomer with > 99 % probability (Tables S4, S5 and S10 in Supp. Info.). Elucidation of the C29-C39 stereocluster, started with assignment of the oxirane ring at C35-C36 as *trans* on the basis of the observed $^3J_{H35,H36}=1.3$ Hz value. Next, the C34-C35 and C36-C39 stereochemical relationships were assigned using a JBC analysis. Measured values of $^3J_{H34,H35}=8.9$ Hz and $^3J_{H36,H37}=7.7$ Hz forwarded to an analysis of the ROESY experiment (Figures 3 and 4), which resulted in two *erithro* configurations. The slightly large value $^3J_{H36,H37}=7.7$ Hz is probably due to the particular geometry of the oxirane ring that orientates H36 and H37 in an intermediate position between an eclipsed and a staggered conformation (Figure 4). In addition, a QM-NMR study to join both sides of the oxirane was undertaken on truncated models, permitting a single stereoisomer to be predicted (29*S**, 31*S**, 32*R**, 33*S**, 34*R**, 35*S**, 36*R**, 37*S**, 39*R**) with a DP4 probability > 99% (Tables S6, S7 and S10 in Supp. Info.). Likewise, the 43*S**, 44*S**, 45*R**, 46*S**, 47*S**, 48*S** relative configurations were determined within the next stereocluster by JBC analysis, including a conformational equilibrium along the C44-C45 bond. The relative configuration of the C47-C48 rotamer was proposed by the absence of ROE between C46 and C49 (Figure 4).¹⁹ Finally, stereochemical assignments of the 51-54 moiety were done using Kishi's NMR universal databases as well as DFT calculations, because chemical shift isochronism of H53_a and H53_b precluded a JBC analysis.²⁴ Both approaches provided 51*R**, 52*S**, 54*S** relative configurations with DP4 probability > 99% (Tables S8- S10 in Supp. Info.).

At this point, the relative configuration of each stereocluster was assigned. However, joining all of them and the single asymmetric centres at C2 and C58 was unresolved. The structural characteristics of PA make a JBC analysis inappropriate for this task.^{25,26} Essentially, the predominantly acyclic nature of PA and the large number of single bonds present in PA, the conformational landscape of this molecule is clearly beyond the capabilities of the approaches most often used.^{23,27} Nevertheless, the recently published *J*-DP4 methods use both chemical shifts and geometrical information obtained from $^3J_{HH}$ values.²⁸ Importantly in this study, the so-called *iJ*-DP4 method only makes use of a subset of candidate conformations compatible with experimental $^3J_{HH}$ to constrain conformational sampling, thus greatly reducing the number of possibilities. For the first time, in this case all $^3J_{HH}$ and $^2J_{CH}$ values that were indicative of non-equilibrium situations along single bonds were incorporated into a restricted conformational search for all possible 64 candidate diastereoisomers (seven

Figure 4. NMRJ-based configurational analysis of prorocentric acid. H and L sub-indexes refer to the low and high chemical shifts for each methylene proton respectively. Red arrows indicate observed ROE correlations.

stereoclusters). This was a key stage because one of the main sources of error in QM-NMR calculations derives from the limited capabilities of computational methods to find the real conformation of the target molecule in complex situations and in addition we greatly reduced the number of possible conformations to be calculated.

Next, NMR chemical shift calculations for each isomer were carried out at the B3LYP/6-31G(d,p) level of theory using GIAO as described for *J*-DP4. Calculation of *J*-DP4 probability resulted in a best match for the 2R*, 5S*, 6R*, 7S*, 8R*, 9R*, 10S*, 15R*, 16S*, 17S*, 18S*, 19S*, 21R*, 22R*, 23S*, 25R*, 29S*, 31S*, 32R*, 33S*, 34R*, 35S*, 36R*, 37S*, 39R*, 43S*, 44S*, 45R*, 46S*, 47S*, 48S*, 51R*, 52S*, 54S*, 58S* relative configuration shown in figure 1 with 98.9% probability (H + C *J*-DP4) (Tables S10 to S27 in Supp. Info.). Regarding the high *J*-DP4 probability value obtained and the small chemical shift differences between some candidates, it has to be remembered that DP4 methods severely penalize outliers. This provides them the ability to differentiate between similar candidate structures (i.e. epimers) but it is also responsible for the known tendency to overstate the final probability in some cases. Still, DP4 has been successfully used in structure elucidation of complex polyketides.^{15,16,29,30} Moreover, *J*-DP4 seems to address the previously mentioned problems.³¹ In the original *J*-DP4 development study, the few incorrectly assigned structures (< 5%) didn't give high confidence on such erroneous results. Nevertheless, due to the extremely complexity of PA, our structural proposal would demand a synthetic approach to be unambiguously confirmed.

Some high molecular weight polyoxygenated compounds are among the most potent toxins known, making them extensively used as pharmacological tools. Okadaic acid and palytoxin effects on cellular physiology have been studied in a variety of cells, including neurons.^{32,33} We first tested the possible effect of PA on neuronal survival using cultured cerebellar neurons as an established experimental model. The exposure of cultured cerebellar

neurons during 24 h to increasing concentration of PA, resulted in a progressive degeneration of neurites and cell bodies starting at 0.5 μ M PA, with an EC50 $\approx$ 1.7 μ M PA (Figure S37). Other cultured neurons, such as cortical neurons, underwent a neurodegeneration similar to that of cerebellar neurons following 24 h exposure to 5 μ M PA (data not shown), but cortical neurons did not degenerate following 24 h exposure to 1 μ M PA, and maintained an intact morphology (Figure S38). The robust electrical activity of cortical neurons allowed us to study the effect of PA on this exclusive neuronal property by recording extracellularly the electrical activity of cortical neurons grown on Micro-Electrode Arrays (MEAs), an emerging, prominent technology for the rapid screening of the pharmacological and toxicological effects of new compounds, preserving neuronal integrity and allowing for long-time recordings.³⁴⁻³⁶ A representative recording of neuronal electrical activity from 10 active MEA electrodes before and after neuronal exposure to 1 μ M PA is shown in Figure 5.

Spontaneous electrical activity of cultured cortical neurons is usually characterized by the occurrence of single action potentials or spikes, and the organization of spikes in bursts (≥ 3 spikes) or network bursts (Figure S39).³⁷ Untreated cortical neurons showed each one of these components of electrical activity, altogether defining a specific pattern with a periodicity of approximately 1 NB/min (Figure 5 and Table 1). Exposure to 1 μ M PA for 18 or 24h produced significant changes in this activity pattern by reducing by more than 50% the number of bursts while increasing by more than 250% their duration. Furthermore, PA exposure increased the inter-burst interval (IBI), and the frequency of shorter NB. This pattern of electrical activity occurred with a higher periodicity of approximately 2.9 NB/min. Interestingly, the proportion of active electrodes/total carrying either spikes or bursts did not change at any time throughout the exposure to 1 μ M PA, nor the spiking frequency in each electrode (Table S28). Furthermore, the number of spikes in each burst remained constant throughout the exposure to PA. Altogether these observations are consistent

with the reshaping of the neuronal electrical activity by PA, in the absence of morphological or functional damage, related to neuronal plasticity.

Figure 5. Effect of 1 μM PA on the network electrical activity of cortical neurons in culture. Representative raster plots of 10 electrodes (top panels) and time histograms of network spikes (total spikes/s, bottom panels) from the same cortical network (a) before (untreated) and (b) after exposition to 1 μM PA for 24 h.

Table 1. Analysis of the spontaneous bursting electrical activity of cultured cortical neurons after 18–24h exposure to 1 μM prorocentric acid (PA)

Burst parameters	Control	PA (1 μM) 18–24h
Bursts/min	10.6 ± 1.2	4.1 ± 0.3 ^[b]
BD (ms) ^[a]	597 ± 68	1529 ± 169 ^[b]
IBI (s) ^[b]	10.1 ± 1.9	17.6 ± 1.3 ^[b]
NB/min ^[c]	0.9 ± 0.2	2.9 ± 0.1 ^[a]

[a] BD = Burst duration. [b] IBI = Inter-Burst-Interval. [b] NB = Network bursts. [d] $P \leq 0.05$. Values are mean $\pm$ SEM (n=40–60 electrodes from 2 independent replicates).

In conclusion, we have isolated, chemically characterized and explored the toxicological and physiological effects in cultured neurons of PA, a structurally unique compound. We have explored for the first time the use of $^{23}\text{J}_{\text{CH}}$ together with $^{31}\text{J}_{\text{HH}}$ and chemical shifts in the calculation of the J -DP4 probability. This new approach greatly improved the prospect of finding the right conformation and highly reduced computational times, extending the usage of this kind of calculations to very large and complex molecules that otherwise have been unapproachable. Our results indicate that cultured cortical neurons exposed to subtoxic concentrations of PA (1 μM) for up to 24h, undergo complex changes of their electrical activity, and therefore of their initial physiological steady-

state. Interestingly, those changes appear to be very different from those observed in neurons exposed to another microalgal molecule, the amnesic shellfish toxin domoic acid (DA). When used at subtoxic concentrations, DA induced an increase in spikes/sec and burst/min after both short and long times of exposure to it,^{34,35} while PA decreased the number of burst/min and did not modify the number of spikes/sec. The changes observed for subtoxic concentration of DA are possibly related to an abnormal modulation of the neuronal learning mechanisms eventually leading to a facilitation of epileptic seizures as observed in animals and humans exposed to low concentration of DA,^{38,39} and hopefully further studies will identify the functional pathways targeted by PA, providing a full picture of the importance of the electrophysiological changes we observed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, spectroscopic data, as well as computational details, coordinate files, experimental and calculated NMR data, correlation plots and values for DP4 and J-DP4 methods (PDF)

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Notes

The authors declare no competing financial interest.

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