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Acuminolide A: Structure and Bioactivity of a New Polyether Macrolide from Dinoflagellate *Dinophysis acuminata*

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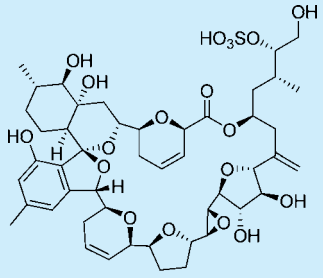
Supporting Information

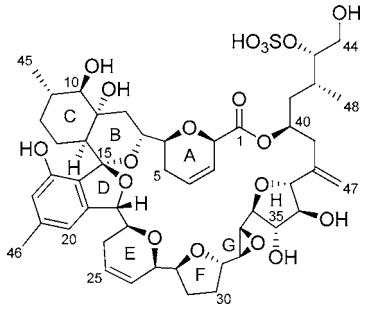
ABSTRACT: Acuminolide A (**1**), along with pectenotoxin II (PTX-2), dinophysistoxin I (DTX-1), okadaic acid (OA), and 7-*epi*-PTX-2 *seco* acid, was isolated from a large-scale cultivation of the dinoflagellate *Dinophysis acuminata*. The new 33-membered macrolide **1** was characterized by detailed analysis of 2D NMR and MS data. Its relative stereochemistry was elucidated on the basis of ROESY correlations and *J*-based analysis. In contrast to the other well-known toxins that were isolated, **1** showed no cytotoxicity against four cancer cell lines but caused potent stimulation of actomyosin ATPase activity.



acuminolide A

Several marine dinoflagellates are known to produce a variety of structurally complex and biologically toxic secondary metabolites.[1] The *Dinophysis* species is a well-known example of such harmful dinoflagellates. This genus has received special attention because it is economically damaging to the bivalve culturing industry through diarrhetic shellfish poisoning (DSP).[2] For a long time, in-depth study of this species was hampered due to the unavailability of laboratory strains. However, after the successful culturing of *D. acuminata* in 2006, much research has been now conducted on this species.[3] In particular, well-known toxins were identified from the culture of *D. acuminata*, including pectenotoxin II (PTX-2) which is isolated primarily from the *Dinophysis* species. This compound is a promising potential anticancer agent because it promotes apoptosis of p53-deficient cancer cells.[4] For this reason, our group aimed to obtain sufficient amounts of PTX-2 from a substantial culture of *D. acuminata* for a study on the mechanism of action of PTX-2. To this end, we successfully isolated 120 mg of PTX-2 from a 3000 L culture of *D. acuminata*. While separating other toxins, an interesting macrolide, acuminolide A (**1**), was isolated with the other known toxins, dinophysistoxin 1 (DTX-1), okadaic acid (OA), and 7-*epi*-PTX-2 *seco* acid. Unlike the known toxins, **1** showed no cytotoxicity on four cancer cell lines but stimulated actomyosin ATPase activity. Here, we describe the structural characterization and bioactivity of acuminolide A (**1**, 4.1 mg).

Harvested cells by continuous centrifugation were extracted with MeOH and then partitioned between H_2O and CH_2Cl_2. The organic fraction was chromatographed on reversed phase silica and Sephadex LH-20 columns to yield the cytotoxic fractions. Compound **1** was isolated from the polar cytotoxic fraction using a gradient eluting HPLC.



On the basis of the negative HR-ESI-MS spectrum, **1** was found to have the molecular formula $C_{48}H_{64}O_{19}S$ ($[M - H]^-$ = 975.3682, Δ = 0.2), which corresponded to 17 degrees of unsaturation. The strong absorptions at 3423 and 1737 cm^{-1} in the IR spectrum indicated the presence of hydroxyl and carbonyl groups. Additionally, a sulfate ester group was suggested from the signals at 1210 and 1046 cm^{-1} in the IR spectrum and by the sulfur atom in the molecular formula. This deduction was confirmed by a fragment of *m*/*z* 97 in the negative MS/MS spectrum. Following this, NMR experiments were performed to elucidate the fine structure of the compound. The NMR experiments were measured in a solvent mixture of 50% D_2O and 50% ACN-d_3 to give well-resolved resonances in the ^1H NMR spectrum. The ^1H and HSQC NMR spectra of **1** showed 40 carbon signals in the form of three methyl (two doublets and one singlet), nine methine (three sp^3 and six sp^2-hybridized), 17 oxymethine, 10 methylene, and one *exo*-methylene groups. The ^{13}C NMR spectrum also showed eight unassigned quaternary

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carbons, including one carbonyl and one ketal. Specifically, two HSQC correlations (6.53/117.3 ppm and 6.52/113.8 ppm) and four sp^2-hybridized quaternary carbons were indicative of a benzene ring, which was supported by UV absorption bands at 220 nm (ε 15200) and 276 nm (ε 3690). The remaining four sp^2-hybridized methines implied the presence of two disubstituted double bonds. The above information led to the conclusion that **1** possesses nine cyclic moieties.

Careful interpretation of the COSY and TOCSY spectra established the presence of four spin systems (I, II, III, and IV) as indicated by the bold lines in Figure 1. Spin systems I and II are

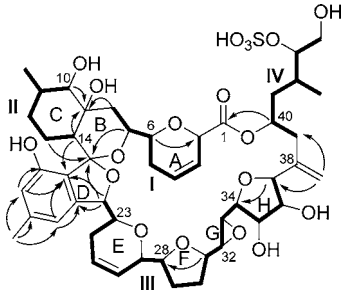

Figure 1. COSY and selected HMBC correlations of **1**.

closely linked, leading to their concurrent identification. The broad H-2 proton signal at δ_H 4.83 is coupled with the signals of four protons: one olefinic proton, H-3; one allylic proton, H-4; and two homoallylic protons, H-5. The HMBC correlation of the oxymethine proton H-6 with C-2 established the dihydropyran structure (A), and further HMBC correlations of the methyl proton H-45 and three neighboring carbons aided the assignment of the partial structure II. In the HMBC spectrum, one terminal proton of substructure I and two terminal methine protons (δ_H 2.99 and 2.18 ppm) of substructure II correlated to the hydroxyl quaternary carbon at C-9. These correlations enabled the assignment of a cyclohexane ring (C) in substructure II, as well as the connection between the two partial structures, I and II. Furthermore, the acetal carbon extending from ring C correlated with the oxymethine proton H-7 through an ether linkage, identifying a tetrahydropyran (B) fused to ring C, named octahydroisochromene as evidenced by a strong HMBC correlation of H13/C-15 and H-7/C-15.

Partial structure III was characterized by consecutive proton couplings from H-22 to H-37 and the chemical shifts of 11 oxymethine carbons. Among them, the broad signal of the proton on C-27 coupled with four proton signals, in a highly similar manner to the COSY coupling pattern for H-2 in partial structure I, immediately indicating a close resemblance between the structures. The ROESY correlation between H-23 and H-27 then revealed another dihydropyran unit (E) which is identical to ring A. Two adjacent carbons at C-32 and C-33 showed more upfield shifted signals with larger J_{CH} values (*ca.* 170 Hz) than are usual for oxymethine carbons. In general, these unusual spectral characteristics are typical for highly strained moieties, such as an epoxide group. Accordingly, these data implied the presence of an epoxide unit (G) in partial structure III. Furthermore, this unit could be also assigned a *trans*-disubstituted configuration due to a small coupling constant between H-32 and H-33 (J_{HH} = 2.5 Hz).[5] Next, two weak HMBC correlations of H-31/C-28 and H-37/C-34 led to the assignment of two tetrahydrofuran moieties F and H, respectively. The structures of F and G were also supported by ROESY signals of H-29a/H-31 and H-34/H-36. Thus, the connections of four ether rings via single bonds are incorporated in spin system III.

This substructure is then connected to the acetal carbon C-15 of ring B, part of substructure II, through an ether bond, which was identified by the HMBC correlation of the terminal proton H-22 with C-15. Together with this linkage, extensive HMBC correlations of H-22/C-16, H-22/C-20, H-22/C-21, H-20/C-21, H-20/H-16, H-46/C-19, and H-14/C-16 revealed a 5-methyl-1,3-isobenzofuran moiety between ring B and C-22. On the other hand, the other terminal carbon of partial structure III was connected to partial structure IV via the as yet unassigned *exo*-methylene group, as evidenced by the HMBC correlations of the two *exo*-methylene protons, H-47, with each terminal carbon of the two partial structures, III and IV. A broad oxymethine proton at δ_H 5.21 in partial structure IV also showed an HMBC correlation with the carbonyl carbon corresponding to an ester bond. The absence of further correlations with the carbonyl carbon prohibited further connectivity, but one remaining degree of unsaturation of **1** allowed the carbonyl carbon to connect with the unbound oxymethine carbon C-2 to constitute a macrolide. This could also be supported by weak ROESY signals of H-3/H37 and H-3/H-47. Thus, the majority of the spin system was now assigned and the macrolide structure had been deduced.

The positions of the remaining six hydroxy and one sulfate groups were assigned based on the carbon chemical shifts. In particular, the attachment of the sulfate ester group on C-43 was determined from a characteristic downfield shifted carbon and proton signals. The planar structure of **1** was confirmed by MS/MS data, following treatment of **1** with NaOMe in MeOH. The observed fragments are rationalized as the ester-cleaved derivative of **1** (Figure S1, Supporting Information).

Following the elucidation of the structure, the relative stereochemistry of **1** was determined on the basis of the ROESY correlations and *J*-based configuration analysis.[6] Measurement of homo- or heteronuclear coupling constants for the *J*-based analysis was achieved from the DQCOSY and HECADE spectra.[7] Initially, the configuration of the relatively rigid octahydroisochromene skeleton was assigned by ROESY correlations. The ROESY cross peaks of H-10/H-12a, H-10/H-14, and H-12a/H-14 on ring C indicated a chair conformation for cyclohexane, and further strong ROESY signals of H-11/H-8b, H-7/H-8b, and H-7/H-13a showed an unusual configuration illustrated in Figure 2A: a *cis*-fusion of ring C with ring B, with a twist-boat conformation. Next, the isobenzofuran connected

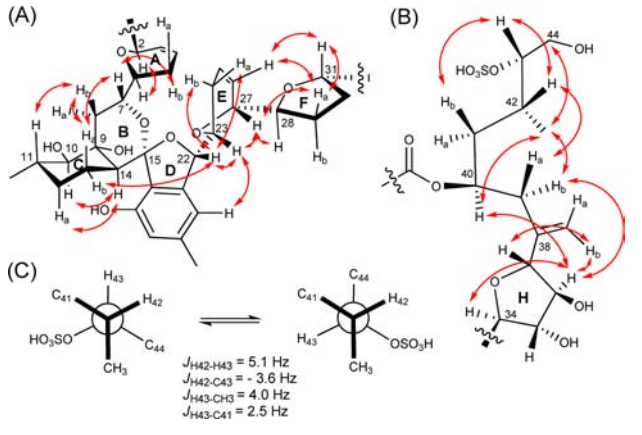

Figure 2. Relative stereochemistry of (A) C-2−C-31, (B) C-34−C44, and (C) C-42−C43 in **1**.

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spirally to C-15 was deduced to be positioned with the phenyl group pointing down and the furan up, because of the steric hindrance imposed by the octahydroisochromene. This configuration was corroborated by a strong ROESY correlation of H-13b/H-22 as well as by an intense HMBC signal of H-14/C-16, resulting from a nearly zero dihedral angle between H-14 and C-16.

The relative orientation of the D/E rings was determined by ROESY cross peaks of H-20/H-23, H-22/H-24a, and H-22/H-24b, along with a small coupling constant (2.5 Hz) between H-22 and H-23. Similarly, the orientation of ring F could be established by the unambiguous ROESY signals of H-26/H-29a, H-26/H-31, H-27/H-28, and H-27/H-29a. Unlike the orientation of ring E, that of ring A could not be clarified from the two ROESY correlations of H-6/H-8a and H-5b/H-7, or a large coupling constant between H-6 and H-7, which can adopt both the (*R*)- or (*S*)- forms on C-6. Instead, the lack of a ROESY signal between H-5 and H-8 for the preferred 6-(*S*) configuration shows an *anti* relationship for C-5 and C-8. The configurations of carbons 2 to 31 (2*R**, 6*S**, 7*R**, 9*R**, 10*R**, 11*S**, 14*S**, 15*R**, 22*S**, 23*S**, 27*R**, 28*S**, and 31*S**) in Figure 2 A could be supported by the weak ROESY correlations of H-5b/H-28 and H-7/H-24b.

The ROESY spectrum indicated that H-37 and H-47 in spin system C-34−C-44 interrelated with H-3 in the C-2−C-31 system. On the basis of this observation, the configuration of ring H was established by the ROESY correlation of H-34/H-36 and the large coupling constants for the two protons of H-35 and H-36. Additional ROESY signals of H-37/H-47b, H-39a/H-47a, H-36/H-40, and H-38/H-40 allowed us to determine the relative stereochemistry for C-34−C-40 (34*R**, 37*R**, and 40*S**) as shown in Figure 2 B. The configuration of the chiral centers in the side chain, C-40−C-44, was established by ROESY correlations and *J*-based analysis. For C-42, the ROESY signals of H-39/H-42 and H-40/H-48 allowed its stereochemistry to be assigned as (*R**), which was also confirmed by the *J*-based analysis (Figure S2). The configuration for C-43 could be determined by analysis of the coupling constant values given in Figure 2 C. A medium coupling value (5.1 Hz) between H-42 and H-43 and a medium heteronuclear coupling (−3.6 Hz) between H-42 and C-43 suggested two exchanging conformations which, together with the other two coupling constants, revealed a *threo* relationship with C-42.

Extensive ROESY correlations (H-28/H-33, H-30a/H-33, H-31/H-32, H-32/H-34, H-32/H-35, H-30a/H-33, H-31/H-33, and H-31/H-35) for H-32 and H-33 on the *trans*-epoxide (G) allowed us to deduce a flexible conformational change of the ring (Figure 3). To begin, the medium coupling constant (4.9 Hz) between H-31 and H-32 indicated interchangeable conformations between the *anti* and *gauche* relationships for the two protons. A small heteronuclear coupling constant (1.6 Hz) between H-32 and C-30 showed that H-32 was in the *gauche* arrangement to C-30. The two coupling constants provided the orientation of the conformational motion around the bond of C-31 and C-32. Compared with this, the motion between C-33 and C-34 was considered to be fixed by the observed coupling constants: a small value (2.5 Hz) between H-33 and H-34 indicating that the two protons were *gauche*, and a large value (6.0 Hz) between H-33 and C-35 showing that the two spins were *anti*. Although the observed coupling values do not allow assignment of orientation of the oxygen in the peroxide ring, all observed ROESY signals allowed its determination based on the predicted conformational motion of G, by which the oxygen atom was determined to be oriented upward (32*S** and 33*R**).

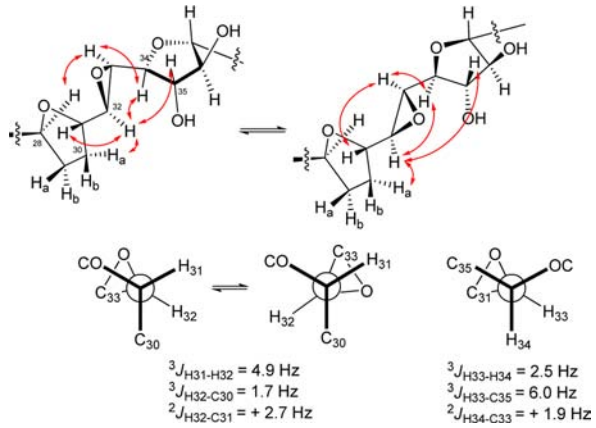

Figure 3. ROESY correlations with H-32 and H-33, and the related coupling constants.

Finally, a ROESY signal of H-2/H-33 supported the relative stereochemistry of **1** that we have assigned. Unfortunately, an attempted Mosher's reaction for the determination of absolute stereochemistry was not successful.

With the total structure and relative stereochemistry of **1** now defined, its bioactivity was investigated. Although isolated from the fraction of *D. acuminata* showing strong cytotoxicity, **1** did not exhibit any cytotoxicity against four different cancer cell lines (K562, U972, HL60, and THP-1). Recently, goniodomin A, a polyether macrolide isolated from dinoflagellate *Alexandrium hiranoi* (*Goniodoma pseudogoniaulax*), was reported as a powerful stimulator of actomyosin ATPase present in skeletal muscles.[8] Thus, taking into account the structural similarity between goniodomin A and **1**, the effect of **1** on actomyosin ATPase found in skeletal muscles was investigated. Interestingly, very similar profiles in ATPase activity were obtained on treatment with **1** as are seen for goniodomin A. As shown in Table S2, at concentrations above 10^{-7} M, the activity of actomyosin ATPase increased with an increase in the concentration of **1**, peaking at 10^{-6} M. The peak value of ATPase activity was 2.5 times higher than that of the control. When the concentration was increased above 10^{-6} M, actomyosin ATPase activity decreased.[9]

In conclusion, **1** is a new 33-membered polyether macrolide isolated from dinoflagellate *D. acuminata*. This compound was found to be composed of two dihydropyrans, two tetrahydrofurans, one dihydroisobenzofuran, one octahydroisochromene, and one epoxide, together with a side chain containing a sulfated substituent. This compound was isolated with potent marine toxins but did not show any cytotoxicity for four cancer cell lines. Instead, **1** showed potent stimulation of the activity of actomyosin ATPase found in skeletal muscles. The dinoflagellate *D. acuminata* is of interest because of its bioactive metabolites. We are currently undertaking a study on efficient cultivation techniques to improve the amounts of useful bioactive metabolites and the relative variations of the constituent toxins according to the cultivation conditions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and spectral data of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(9) Please see Supporting Information for further explanation of ATPase activity.