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Clarification of the C-35 Stereochemistries of Dinophysistoxin-1 and Dinophysistoxin-2 and Its Consequences for Binding to Protein Phosphatase

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Okadaic acid analogues are well known as protein phosphatase inhibitors and occur naturally in marine shellfish feeding on dinoflagellates of the genus *Dinophysis*, leading to diarrhetic shellfish poisoning of shellfish consumers. Knowledge of the correct structures for these toxins is important in understanding their toxicology, biochemistry, and biosynthesis. We have performed extensive NMR analyses on okadaic acid (**1**), dinophysistoxin-1 (DTX-1), and dinophysistoxin-2 (DTX-2) obtained from natural sources. Consequently, we were able to unambiguously deduce the stereochemistries at C-35 for DTX-1 and DTX-2 based on analysis of NMR coupling constants and NOE interactions. Our results revealed that DTX-2 (**3**) has a stereochemistry opposite to that of DTX-1 (**2**) at C-35. Molecular modeling of the docking of **1**−**3** with protein phosphatase-1 and protein phosphatase 2A (PP2A) suggested that the reduced affinity of DTX-2 for PP2A may be due to the newly defined stereochemistry at the 35-methyl group. The implications of these findings for biosynthesis and toxicology are discussed.

Introduction

Okadaic acid (*1*, *2*) and its analogues, originating from certain species of marine algae, can occur in shellfish throughout the world and are associated with "diarrhetic shellfish poisoning"-(DSP)[1] (*3*). Some of these analogues bind to and potently inhibit protein phosphatase-1 and -2A (PP1 and PP2A), leading to the widespread use of okadaic acid in biochemical studies of the role of PPs in biological systems (*4*). In connection with recent studies directed toward the isolation and characterization of okadaic acid analogues from *Dinophysis acuta* (*5*), we had occasion to critically compare published NMR chemical shifts for OA, DTX-1, and DTX-2 (*2*, *6*). This analysis identified some variations in the NMR shifts reported for the C-30−C-38 portion of DTX-1 compared to those for DTX-2, which, if correct, suggested that the C-35 configurations of these compounds were not identical. This conclusion was contrary to the widely held belief in the literature and reviews (e.g. refs *4* and *7*) that the C-35 stereochemistry of DTX-1 (**2**) is the same as that of DTX-2 (i.e., **4**) (Figure 1).

In 1982, Murata et al. reported the structure of DTX-1 on the basis of NMR analysis (*2*). The couplings that H-35 showed to the H-36 methylene protons when the 35-methyl-group protons were irradiated in a decoupling experiment were reported as being 3.2 and 11.2 Hz (*2*), which is consistent with an axial−equatorial and an axial−axial coupling, respectively. This coupling constant data indicates that H-35 is axially oriented and the 35-methyl is equatorially orientated in DTX-1, and that DTX-1 is therefore 35*R*-methyl-**1** (**2**). However, Murata et al. (*2*) described DTX-1 as 35*S*-methyl-**1** (a structure corresponding to **5**) in their text.

The structure of DTX-2 was reported in 1992 by Hu et al. (*6*). These authors presented NMR NOE evidence that the 35-methyl group in DTX-2 was axially orientated (Figure 1 in ref *6*), making it 35*S*-methyl-31-desmethyl-**1** (**3**). The 35-stereochemistry of DTX-2 (*6*) therefore appeared to correspond to the 35*S*-stereochemistry reported for DTX-1 (*2*). Consistent with this, the 35-Me in DTX-1 was also reported to show NOE correlations analogous to those observed for DTX-2 (Table 1 footnote d in ref *6*), indicating that it too was axial and that DTX-1 and DTX-2 possessed the same stereochemistry at C-35. A footnote in a 1993 review (*3*), however, states that the C-35

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[1] Abbreviations: DSP, diarrhetic shellfish poisoning; DTX, dinophysistoxin; OA, okadaic acid; PDB, Protein Data Bank; PP, protein phosphatase.

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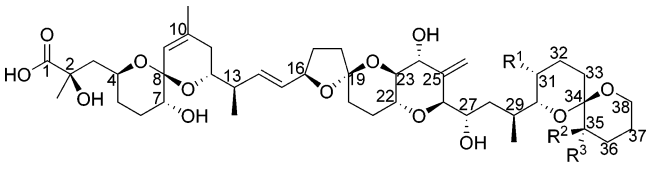

		R^1	R^2	R^3	31	34	35
1	Okadaic acid	CH_3	H	H	*R*	*S*	-
2	DTX-1	CH_3	CH_3	H	*R*	*R*	*R*
3	DTX-2	H	H	CH_3	-	*R*	*S*
4		H	CH_3	H	-	*R*	*R*
5		CH_3	H	CH_3	*R*	*R*	*S*

Figure 1. Structures of okadaic acid analogues discussed in the text and their stereochemistries at the 31-, 34-, and 35-positions. The correct structures are shown for okadaic acid (**1**), dinophysistoxin-1 (**2**), and dinophysistoxin-2 (**3**). Structure **4** shows the commonly depicted, but incorrect, structure for dinophysistoxin-2, and structure **5** is hypothetical.

stereochemistry of DTX-1 is 35*R*, and not 35*S* as originally reported (*2*). In 1998, Sasaki et al. (*8*) determined the absolute stereochemistry of DTX-1 by NMR analysis of a chiral amide derivative (at C-1) and confirmed the stereochemistry of DTX-1 to be 35*R*-methyl-**1** (i.e., **2**). No mention was made in the review (*3*), or by Sasaki et al. (*8*), of the potential implications of the proposed revision of the C-35 stereochemistry of DTX-1 with respect to the structure of DTX-2. Specifically, the question that arises is whether the structure of DTX-2, which was considered to possess the same C-35 configuration as DTX-1 (*6*), also required revision.

Given these uncertainties, and the importance of these compounds in shellfish poisoning and biochemical studies, we have undertaken NMR analysis of okadaic acid, DTX-1, and DTX-2 in order to clarify their C-35 stereochemistries. Here, we report detailed analyses of one- and two-dimensional NMR data in several solvents, including DEPT135, g-HSQC, g-HMBC, COSY, TOCSY, NOESY, and/or ROESY, and ^{1}H homonuclear decoupling difference NMR experiments, which show that the 35-methyl group of DTX-2 is axially oriented and that the 35-methyl group of DTX-1 is equatorially orientated. Our analyses confirm that the stereochemistry of DTX-1 (**2**) is as depicted in Figure 1 but reveal that DTX-2 should be represented as **3**, rather than as **4** as has been the case in some of the literature published to date. This finding has implications for structure−activity studies, immunoassay development, the biosynthesis of okadaic acid analogues, and for studies on the total synthesis of these compounds.

Materials and Methods

General. Okadaic acid was purchased as the sodium salt from Sapphire Bioscience, Redfern, Australia. Flash chromatography was performed on Lichroprep RP-18 (40−63 μm, Merck, Germany) by gradient elution. Monitoring of fractions from flash chromatography was performed by LC-MS with an LCQ classic mass spectrometer (Finnigan MAT, San Jose, CA) as described for phytoplankton by Miles et al. (*9*).

Isolation of DTX-1. DTX-1 was isolated from *Dinophysis acuta* harvested from Sognefjord, Norway (*9*). DTX-1 was present in fractions 9−11 from the flash column chromatography of the ethereal extract described by Miles et al. (*9*) for the purification of PTX-12 (which was present in fractions 17−21). The dichloromethane extract from the Sognefjord algal sample (*9*) was also treated in an identical manner (chromatography on alumina followed by flash chromatography) to provide a fraction also containing DTX-1. Both DTX-1-containing fractions were combined and purified by flash column (320 × 10 mm) chromatography with an FPLC pump system (Pharmacia, Uppsala, Sweden) consisting of a gradient programmer GP-250, dual P-500 pumps, and a dual path UV detector (UV-2 optical unit and its control unit) eluted with 30% MeCN (40 mL), followed by a linear gradient from 30−100% MeCN (over 680 mL) at 6 mL/min, and 10-mL fractions were collected. DTX-1, present in fractions 6−13, was further purified on the same system using a series of linear gradients containing the following: (A) 10 mM ammonium formate and (B) 9:1 MeCN−10 mM ammonium formate. The gradient consisted of 50 mL of 20% B; 50−250 mL of 20−50% B; 250−260 mL of 50−100% B; followed by 90 mL of 100% B, at 6 mL/min, and fractions of 10 mL were collected. Fractions 25−29 were concentrated in vacuo to provide DTX-1 as a colorless solid (6.6 mg).

Table 1. ^{13}C NMR Chemical Shifts for C-35−C-37 of DTX-1 (2) and DTX-2 (3) (from ref *6*), and for the Equivalent Atoms in *trans*-1-methyl-4-*t*-butylcyclohexane (6) and *cis*-1-methyl-4-*t*-butylcyclohexane (7) (*18*) in $CDCl_3$

atom	δ **2**	δ **3**[a]	Δ (δ **2** − δ **3**)	atom	δ **6**	δ **7**	Δ (δ **6** − δ **7**)
C-35	39.0	35.9	3.1	α	32.6	27.0	5.6
C-36	27.5	25.7	1.8	β	36.0	33.0	3.0
C-37	26.4	20.0	6.4	γ	27.7	21.4	6.3

Isolation of DTX-2. Two ampoules of processed hepatopancreas extract from Castletownbere were fractionated through alumina as described previously (*10*). The aqueous ammonia fractions were collected and concentrated in vacuo. The residue was further purified by flash chromatography (3 cm i.d., 50 g packing) by stepwise gradient elution with 60% MeOH (30 mL), 80% MeOH, and MeOH (120 mL). The first 30 mL was discarded after which 25 10-mL fractions were collected. Fractions 9−12 contained **3** and were combined and concentrated in vacuo for purification by preparative HPLC. Preparative HPLC was performed on a Gilson 321 HPLC system with a Supelcosil LC-18 DB column (250 × 10 mm, 5 μm) (Supelco, Bellefonte, PA) eluted at 5 mL/min with 56:6:38 of water/MeCN/MeOH containing 0.1% formic acid and 2 mM ammonium formate. Collection of fractions was based on detection by UV absorbance at 200 and 235 nm using a Hewlett Packard Series 1100 G1315A diode array detector. Fractions containing DTX-2, eluting at ca. 9.5 min, were collected and concentrated in vacuo to afford a colorless solid (ca. 3.5 mg).

NMR Analysis. NMR spectra of **1** (1 mg) were obtained from solutions in 0.5 mL of CD_3OD (99.8 + atom% D), $CDCl_3$ (100 atom-% D), and $CDCl_3$ plus ca. 1% CD_3CO_2D (99.5 atom-% D) (all Aldrich, USA) with a Bruker DRX 400 MHz spectrometer. NMR assignments (Table 2) were obtained from examination of ^{1}H, ^{13}C, DEPT135, 1D-TOCSY, COSY, TOCSY, g-HSQC, g-HMBC, ROESY, and NOESY NMR spectral data. NMR assignments of DTX-1 (**2**) (1 mg) and DTX-2 (**3**) (0.4 mg) (Table 2) were obtained from examination of ^{1}H, ^{13}C, DEPT135, 1D-TOCSY, COSY, TOCSY, g-HSQC, g-HMBC, ROESY, and NOESY NMR spectral data. NMR spectra were acquired from 0.5 mL of CD_3OD (99.8 atom% D), 0.5 mL of CD_3CN (100.0 atom-%), and $CDCl_3$ (100.0 atom-%) (all from Aldrich, USA) on a Bruker AV 600 MHz spectrometer with a 5 mm BBI (^{1}H/BB-^{2}H) Broad Band Inverse probe equipped with Z-gradient coils, except that a Bruker AV II 600 MHz spectrometer equipped with a TCI cryoprobe and Z-gradient coils was used to acquire the NMR data for DTX-2 in CD_3CN. Chemical shifts, determined at 298 K, are reported relative to internal C*H*D_2OD (3.31 ppm) and *C*D_3OD (49.0 ppm), C*H*Cl_3 (7.26 ppm), and *C*DCl_3 (77.1 ppm), or C*H*D_2CN (1.94 ppm) and CD_3*C*N (118.7 ppm). Data were processed with Bruker TOPSPIN (version 1.3) and Mestre-C (version 4.5.1). Molecular modeling of **1**−**3** was performed using Chem3D Ultra version 8.0 (CambridgeSoft, Cambridge, MA) by modification of the X-ray crystal structure (*1*) and minimization using the MM2 parameters provided.

Molecular Docking. Molecular docking of okadaic acid and its analogues was carried out using the program GOLD (*11*). Protein data bank (PDB) structure 1JK7 (http://www.rcsb.org/pdb/explore.do?structureId=1jk7, accessed 17 July 2006) for okadaic acid bound to protein phosphatase-1 (PP1) (*12*) was used as the protein target. Additionally, a structure of okadaic acid bound to protein phosphatase 2A (PP2A) recently became available (*13*) (coordinates kindly provided by Y. Shi, Princeton University, Princeton, NJ 08544, USA). In each case, the okadaic acid in the

Table 2. NMR Assignments of Okadaic Acid (1), Dinophysistoxin-1 (2), and Dinophysistoxin-2 (3) in CD_3OD[a]

	okadaic acid (**1**)		DTX-1 (**2**)		DTX-2 (**3**)	
atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	182.8		182.7		180.6	
2	76.3		76.5		76.1	
2-Me	27.9	1.31	27.9	1.31	27.7	1.34
3	46.7	1.65, 1.91	46.6	1.64, 1.94	45.7	1.65, 2.01
4	68.9	4.08	69.0	4.09	69.3	4.08
5	33.4	1.30, 1.89	33.4	1.30, 1.82	33.4	1.34, 1.79
6	28.3	1.66, 1.94	28.2	1.65, 1.95	28.0	1.65, 1.98
7	73.4	3.35	73.4	3.36	73.1	3.37
8	97.5		97.6		97.6	
9	123.8	5.26	123.7	5.27	123.2	5.29
10	139.3		139.4		139.9	
10-Me	23.1	1.72	23.2	1.73	23.1	1.75
11	34.0	1.82, 1.98	34.0	1.84, 1.98	34.1	1.88, 1.96
12	71.8	3.87	71.8	3.86	71.9	3.75
13	43.2	2.35	43.3	2.35	43.3	2.34
13-Me	17.1	1.11	17.1	1.11	16.8	1.07
14	137.2	5.95	137.6	5.97	137.4	5.83
15	132.0	5.50	131.9	5.50	132.3	5.52
16	80.6	4.66	80.6	4.67	80.5	4.62
17	31.5	1.60, 2.21	31.5	1.60, 2.20	31.5	1.61, 2.20
18	38.1	1.85, 2.00	38.1	1.85, 2.01	38.0	1.85, 2.00
19	107.1		107.0		107.1	
20	34.2	1.84−1.87	34.1	1.83−1.90	34.1	1.82−1.89
21	27.7	1.80, 1.90	27.7	1.80, 1.90	27.7	1.79, 1.90
22	71.4	3.63	71.3	3.64	71.4	3.64
23	78.3	3.42	78.2	3.42	78.1	3.40
24	72.1	4.07	72.1	4.06	72.2	4.10
25	147.1		147.1		147.4	
25 $=CH_2$	112.6	5.04, 5.38	112.6	5.05, 5.40	112.1	5.04, 5.36
26	86.4	3.94	86.4	3.95	86.4	3.97
27	66.3	4.08	66.1	4.10	67.0	4.07
28	36.8	0.96, 1.37	36.8	0.96, 1.37	38.6	1.37, 1.44
29	32.3	1.89	32.4	1.89	35.3	1.83
29-Me	16.6	1.05	16.5	1.04	14.4	0.95
30	76.8	3.27	76.5	3.25	75.0	3.42
31[b]	28.8	1.81	28.7	1.81	28.7	1.27, 1.52
31-Me	11.1	0.93	11.1	0.93		
32[b]	27.5	2.00, 1.38	27.5	2.00, 1.41	20.1	1.74, 1.60
33[b]	31.2	1.59, 1.36	26.9	1.98, 1.15	33.4	1.16, 1.68
34	97.0		99.3		99.3	
35	37.0	1.43, 1.61	40.4	1.50	37.4	1.61
35-Me			17.2	0.95	14.7	0.99
36[b]	19.8	1.53, 1.88	28.6	1.63, 1.46	26.7	2.17, 1.29
37[b]	26.5	1.49−1.55	27.5	1.53, 1.64	21.0	1.78, 1.26
38[b]	61.3	3.71, 3.51	60.9	3.66, 3.49	61.4	3.70, 3.51

[a] The assignments for **1**−**3** in $CDCl_3$ and for **2** and **3** in CD_3CN are presented in the Supporting Information. [b] These methylene protons are in the format H_{ax}, H_{eq}.

PDB file was trimmed after position 29, removing the terminal group. Hydrogens were added to the protein and the C-1−C-29 portion of okadaic acid using the program Reduce (*14*) and converted into mol2 format using Open Babel (*15*). Molecular files for the three terminal ring systems (C-30−C-38 of **1**, **2**, and **3**) to be docked to the protein structures were generated and energy minimized using the PRODRG server (*16*). Covalent docking of the okadaic acid and its analogues was carried out using GOLD. A covalent linkage between C-29 of the okadaic acid fragment in the protein target and C-30 of the docked terminal ring system was used to constrain the docking of the ligand to the protein structure. Twenty independent trials were carried out for each ring system using the Chemscore fitness function with default parameters. Chemscore estimates the change in Gibbs free energy on binding using H bonding, metal binding, hydrophobic bond rotation, and clash terms whose coefficients are derived empirically from a set of protein−ligand complexes whose affinities have been measured (*17*).

Results and Discussion

DTX-1 and DTX-2 are generally depicted as having the same orientations for their 35-methyl groups (i.e., **2** and **4**, respectively) (e.g. refs *4* and *7*) and therefore similar terminal ring conformations. However, the published ^{13}C NMR chemical shifts for C-35−C-37 for DTX-1 and DTX-2 in $CDCl_3$ (Table 1) (*6*) are not consistent with this view. If the assignments for these resonances are correct, then either the C-35 stereochemistries of DTX-1 and DTX-2 are not the same or there is a major difference in the conformations of their terminal rings. A comparison of published ^{13}C NMR chemical shifts (*18*) for model analogues **6** and **7** (Figure 2) in $CDCl_3$ with those for **2** and **3** (Table 1) suggests that DTX-1 (**2**) could have an equatorial 35-methyl group and that DTX-2 (**3**) is more likely to have an axial 35-methyl group.

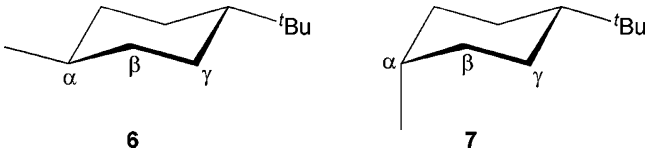

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Figure 2. Structures of *trans*- (**6**) and *cis*-1-methyl-4-*t*-butylcyclohexane (**7**) containing equatorial and axial methyl groups, respectively, for which ^{13}C NMR chemical shift data are presented in Table 1 (*18*).

Detailed NMR studies were required to clarify the stereochemistries of DTX-1 and DTX-2. Okadaic acid was commercially available. DTX-1 was isolated from a bloom of *Dinophysis acuta* from Sognefjord in western Norway. This appears to be the first isolation of DTX-1 from a natural *Dinophysis* bloom, and this source provides a convenient source for isolation of DTX-1 and analogues. DTX-2 was isolated from contaminated mussels from Ireland, where DTX-2 is often the dominant okadaic acid analogue found in shellfish, and where okadaic acid is typically present as a relatively minor component (*19*).

^{1}H and ^{13}C NMR Signal Assignments. Detailed analyses of one- and two-dimensional NMR data, including ^{1}H, ^{13}C, DEPT135, 1D-TOCSY, COSY, TOCSY, g-HSQC, and g-HMBC spectral data determined in CD_3OD (Table 2) and $CDCl_3$ (Supporting Information) afforded complete assignments of the ^{1}H and ^{13}C resonances of OA, DTX-1, and DTX-2. Assignments for DTX-1 and DTX-2 were also determined in CD_3CN (Supporting Information). Typically, $^{1}H-^{1}H$ connectivities were established in COSY and TOCSY experiments, and $^{1}H-^{13}C$ connectivities were identified in g-HQSC and g-HMBC experiments. Methylene proton assignments are reported as $H_{a,b}$ (i.e., stereochemistry not assigned), except that the majority of the H-32−H-38 methylene protons of okadaic acid, DTX-1, and DTX-2 were assigned axial or equatorial orientations (Table 2) on the basis of NOESY (and ROESY, in the case of OA) experiments. The resolution in the ^{1}H axis in the g-HSQC spectra was such that axially and equatorially oriented methylene protons attached to 6-membered ring carbon atoms could be readily distinguished (*20*), providing verification of the stereochemical assignments because, typically, only large ^{2}J (geminal) and $^{3}J_{ax-ax}$ couplings of the order 10−14 Hz and not smaller $^{3}J_{ax-eq}$ or $^{3}J_{eq-eq}$ couplings of the order 3−5 Hz were resolved in the g-HSQC spectra. For example, triplet- and doublet-like signals were observed in the g-HSQC spectrum for the H-33_{ax} (1.98 ppm) and H-33_{eq} (1.15 ppm) signals of DTX-1 (**2**). However, the H-33_{ax} (triplet-like) and H-33_{ax} (doublet-like) signals of DTX-2 (**3**) occurred at 1.16 and 1.68 ppm, respectively (Supporting Information). These observations showed that in DTX-1, H-33_{eq} resonates at higher field than H-33_{ax}, whereas the opposite was the case for DTX-2. The marked variations in chemical shifts of H-33_{ax} and H-33_{eq} can be attributed to the presence of a 31-Me group in DTX-1 (**2**) but not in DTX-2 (**3**).

In CD_3OD, the COSY spectrum of DTX-1 (**2**) included correlations between H-31 (1.81 ppm) and the 31-methyl group protons (0.93 ppm), and between H-35 (1.50 ppm) and the 35-methyl group protons (0.95 ppm). Thereafter, the resonances of nearby methylene protons were identified through correlations observed in TOCSY spectra. For example, the 31-methyl group protons showed TOCSY correlations to H-31, H-$32_{ax,eq}$ (2.00 and 1.41 ppm), and H-$33_{ax,eq}$ (1.98 and 1.15 ppm), whereas the 35-methyl group protons showed correlations to H-35, H-$36_{ax,eq}$ (1.63 and 1.46 ppm), H-$37_{ax,eq}$ (1.53 and 1.64 ppm), and H-$38_{ax,eq}$ (3.66 and 3.49 ppm). Subsequently, ^{13}C resonances were defined by correlations, which these proton showed in an edited (phase-sensitive) g-HSQC spectrum. ^{13}C signal multiplicities (d, t, or q) were verified in a DEPT135 spectrum.

DTX-2 (**3**), which lacks the 31-Me group, has an additional methylene group to be assigned. The H-$31_{ax,eq}$ (1.27 and 1.52 ppm) signals of **3** showed a COSY correlation to H-30 (3.42 ppm) together with TOCSY correlations to H-$32_{ax,eq}$ (1.74 and 1.60 ppm) and H-$33_{ax,eq}$ (1.16 and 1.68 ppm). The resonances of the corresponding ^1H and ^{13}C atoms of OA (**1**), and all other atoms of **1**, **2**, and **3** (Table 2) were established in a similar manner. In general, the ^1H and ^{13}C chemical shifts determined in CD_3OD (Table 2) were similar to those determined in $CDCl_3$ and CD_3CN (Supporting Information).

The most noticeable difference between the ^{13}C resonances established for DTX-1 and DTX-2 in CD_3OD (Table 2) was the occurrence of their C-37 resonances at 27.5 and 21.0 ppm, respectively. These shifts, which are comparable to those reported for these atoms in $CDCl_3$ (Table 1), can be attributed to the presence of an axial 35-methyl group in DTX-2 and an equatorial 35-methyl in DTX-1. Typically, an axial methyl group in a six-membered ring causes the γ-carbon to resonate ca. 6 ppm upfield of where it would resonate if the methyl group was equatorially oriented (Table 1).

β-Substituent effects are less diagnostic, at least with respect to their ability to define the orientation (axial vs equatorial) of a methyl group on the adjacent β-carbon. Although the downfield shifts of C-36 of DTX-1 (**2**) and DTX-2 (**3**) (28.6 and 26.7 ppm, respectively), compared to that of OA (19.8 ppm), are consistent with the presence in **2** and **3** of a methyl group at C-35, they do not unequivocally define the equatorial or axial orientation of the 35-methyl group. The marked difference in the resonance of C-32 (20.1 ppm) of **3** compared to those of **1** (27.5 ppm) and **2** (27.5 ppm) can attributed to the presence in **1** and **2**, but not **3**, of a 31-methyl group.

Other ^{13}C signal assignments in CD_3OD (Table 2) are comparable to those reported for okadaic acid analogues in $CDCl_3$ (*6*, *21*, *22*) and CD_3OD (*23*, *24*), apart from the inadvertent reversal of the C-8 and C-19 ketal carbon assignments in a previous study (*5*). The C-8 resonances of **1**, **2**, and **3** were unambiguously assigned on the basis of g-HMBC correlations that the olefinic H-9 signal of each compound showed to its corresponding C-8, C-11, and the 10-Me carbon (Supporting Information), in agreement with Norte et al. (*21*, *22*).

Only one set of ^1H and ^{13}C NMR signals was observed for OA (**1**), which was purchased in the form of the Na salt, when NMR data was determined in CD_3OD. However, pairs of signals were observed for some of the resonances of atoms in close proximity to the carboxyl group when spectral data was determined in $CDCl_3$. For example, g-HMBC and g-HSQC data identified two 2-Me signals at 1.38 and 1.34 ppm at a ratio of ca. 7:10. This appears to be a consequence of the presence of traces of DCl in $CDCl_3$. The addition of a drop of 1% CD_3CO_2D in $CDCl_3$ afforded a single set of signals (2-Me at 1.38 ppm), attributable to the presence under these conditions of only the free acid form of **1**. g-HMBC data showed that the C-2 and C-3 signals of the Na salt of **1** appeared at 43.3 and 76.6 ppm (2D resolution, $\pm$ 0.5 ppm) compared to 44.3 and 79.0 ppm for the free acid. A correlation from the 2-Me group protons to C-1 (177.9 ppm) was only observed for the free acid and not for the sodium salt.

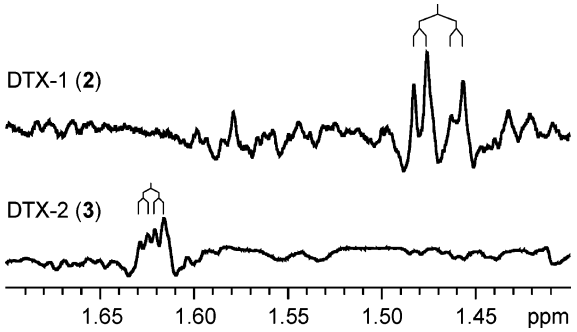

Figure 3. Homonuclear decoupled difference spectra of DTX-1 (**2**) and DTX-2 (**3**) in CD_3CN obtained by decoupling of their respective 35-methyl group proton resonances.

Homonuclear Decoupling-Difference Results. ^1H homonuclear decoupling-difference experiments were performed for DTX-1 and DTX-2 in CD_3CN because the methyl group resonances were well separated in this solvent. Irradiation of the 35-methyl group signal (0.96 ppm) of DTX-2 (**3**) resulted in H-35 (1.55 ppm) appearing as a doublet of doublets (J = 4.7 and 2.5 Hz) (Figure 3). On the basis of the Karplus equation (*25*), this coupling is consistent with H-35 being equatorially oriented and experiencing small equatorial−axial and equatorial−equatorial couplings with H-36_{ax} and H-36_{eq}, respectively, corresponding in each case to dihedral angles of ca. 60°. In contrast, irradiation of the 35-methyl group signal (0.92 ppm) of DTX-1 (**2**) led to H-35 (1.47 ppm) appearing as a doublet of doublets (J = 11.8 and 3.7 Hz) (Figure 3). These couplings, which are similar to those reported by Murata et al. (*2*) for an analogous experiment performed for DTX-1 in $CDCl_3$, are consistent with H-35 being axially oriented. The larger coupling (11.8 Hz) is indicative of a trans-diaxial coupling between H-35 and H-36_{ax} (ca. 180° dihedral angle) and the smaller coupling (3.7 Hz) of an axial−equatorial coupling between H-35 and H-36_{eq} (ca. 60° dihedral angle). These decoupling-difference results unequivocally demonstrate that the 35-methyl group is equatorially oriented in DTX-1 and axially oriented in DTX-2, as shown in Figures 1 and 3.

NOESY Correlations. Structurally significant NOESY correlations observed for OA, DTX-1, and DTX-2 in CD_3OD are presented in Figure 4. (We also observed similar NOESY correlations for these compounds in $CDCl_3$.) Importantly, for DTX-1 (**2**) we observed mutual NOESY correlations between the 31-methyl group protons (0.93 ppm) and H-33_{ax} (1.98 ppm), and between H-33_{ax} and the 35-methyl group protons (0.95 ppm). The H-38_{eq} proton of **2** showed NOESY correlations to H-37_{ax} (1.53 ppm) and H-37_{eq} (1.64 ppm), whereas H-38_{ax} (3.66 ppm) showed NOESY correlations to H-36_{ax} (1.63 ppm), H-37_{eq} (1.64 ppm), H-30 (3.25 ppm), and the 29-Me group (1.04 ppm). In DTX-2 (**3**), H-33_{eq} (1.68 ppm) showed a NOESY correlation to the 35-methyl group protons (0.99 ppm), which in turn showed a NOESY correlation to H-37_{ax} (1.78 ppm), whereas H-38_{ax} (3.70 ppm) showed NOESY correlations to H-36_{ax} (2.17 ppm), H-37_{eq} (1.26 ppm), H-30 (3.42 ppm), and the 29-Me group (0.95 ppm). The foregoing NOESY correlations are again consistent with the 35-methyl group being equatorially oriented in DTX-1 and axially oriented in DTX-2 (Figure 4).

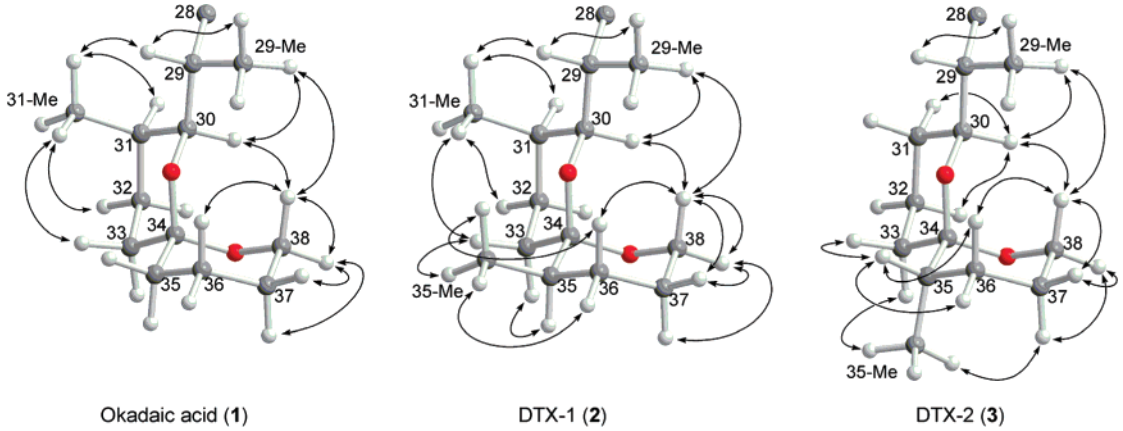

Figure 4. NMR NOE correlations observed for the F- and G- (terminal) rings of okadaic acid (**1**), dinophysistoxin-1 (**2**), and dinophysistoxin-2 (**3**) in CD_3OD. The structures shown are the minimized structures predicted by molecular modeling.

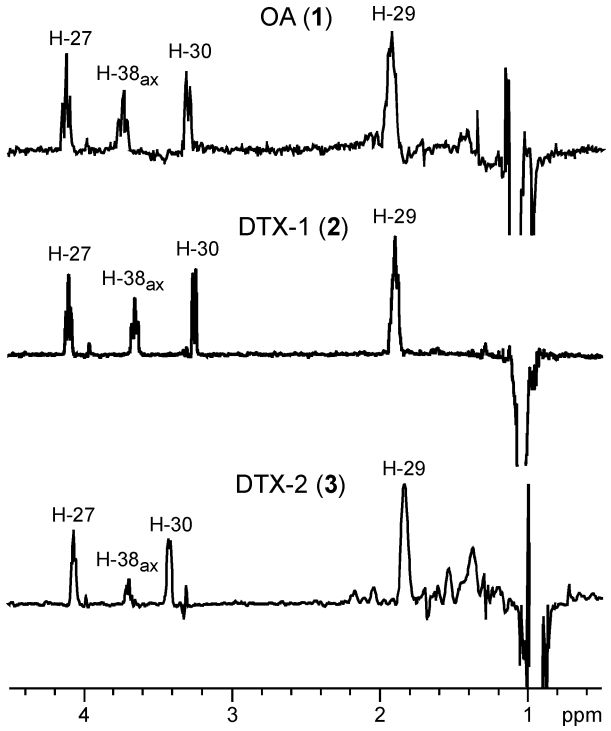

Figure 5. Slices from NOESY NMR spectra of okadaic acid (**1**), DTX-1 (**2**), and DTX-2 (**3**) in CD_3OD showing NOE correlations arising from the 29-methyl groups of these compounds.

The 31-methyl groups of OA and DTX-1 showed NOESY correlations to H-29, H-31, H-32_{eq}, and H-33_{ax}. Additionally, in each of OA, DTX-1, and DTX-2, the 29-methyl group showed NOESY correlations to H-27, H-29, H-30, and H-38_{ax} (Figure 5). These correlations show that in each of these compounds, the preferred conformation in the vicinity of C-29 and the stereochemical relationship between their two terminal rings were similar. This demonstrates that the C-34 stereochemistries of these compounds are the same and that epimerization at this and other nearby carbon atoms has not occurred during isolation and NMR analysis. However, our finding that some of the terminal-ring protons of DTX-1 and DTX-2 exhibited distinctly different NOEs is attributable to the presence of equatorial and axial 35-methyl groups, respectively, in these compounds.

The internuclear distances between H-38_{ax} and H-30, and between H-38_{ax} and the 29-methyl group of OA (**1**) derived from X-ray crystallography (*1*) in the solid state are both 2.6 Å. The detection of mutual NOESY correlations between these protons in **1**, **2**, and **3** (Figure 5) shows that the preferred solution conformation of the C-29−C-38 regions of these molecules are similar to each other and to the solid-state conformation determined for OA (**1**), and justifies the use of the X-ray crystal structure to generate starting conformations for molecular modeling of OA, DTX-1, and DTX-2.

We have shown that OA, DTX-1, and DTX-2 share the same relative configuration at all chiral centers except for C-35, but our NMR data cannot establish the absolute stereochemistries. However, that DTX-2 (**3**) co-occurs in *Dinophysis* together with OA (**1**) and that OA (**1**), DTX-1 (**2**), and DTX-2 (**3**) all inhibit PP2A with similar potencies strongly suggests that **2** possesses the same absolute stereochemistry as that established for **1** (*2*) and **2** (*8*) at their common chiral centers (Figure 1).

Docking with PP1 and PP2A. Molecular docking (Figure 6) was used to investigate the effect of the various substitutions to the binding of the toxin to its protein targets, protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A). The X-ray crystal structure of okadaic acid bound to PP1 has previously been determined ((*12*), PDB 1JK7), and this was used as a starting point to model the binding of DTX-1 and DTX-2. More recently, the 3D structure for okadaic acid bound to PP2A has been reported (*13*), and the coordinates for this structure were obtained from the authors.

As only differences in the binding of the distal terminus of the toxin (rings F and G) were being investigated, a covalent docking protocol was established. The okadaic acid present in the crystal structure was truncated after C-29, and a set of ligands were constructed consisting of only the terminal group of the toxin (C-30 onward). A covalent bond was defined between C-29 of the residual ligand in the protein−ligand complex and C-30 of the terminal group to be docked. Full flexibility of this bond was maintained. To verify the docking procedure, the terminal group from okadaic acid (**1**) was also docked to the protein structure (Figure 6A and D), resulting in a binding mode identical to that observed in the crystal structure of okadaic acid bound to either PP1 or PP2A. Docking of both the DTX-1 and DTX-2 resulted in a similar mode of binding (Figure 6B, C, E, and F). No significant shift was observable in the ring structure or position of the 31-methyl group in DTX-1 (**2**) docked to PP1 (Figure 6B). Also for PP1, the 35-methyl groups in DTX-1 (**2**) and DTX-2 (**3**) were both orientated toward the solvent, and no interaction with the protein was observed for 35-methyl groups for either DTX-1 (**2**) or DTX-2 (**3**) (Figure 6B and C). These results suggest that the inhibitory potency of DTX-2 toward protein phosphatase-1 should be similar to those of okadaic acid and DTX-1. The modeling study suggests that

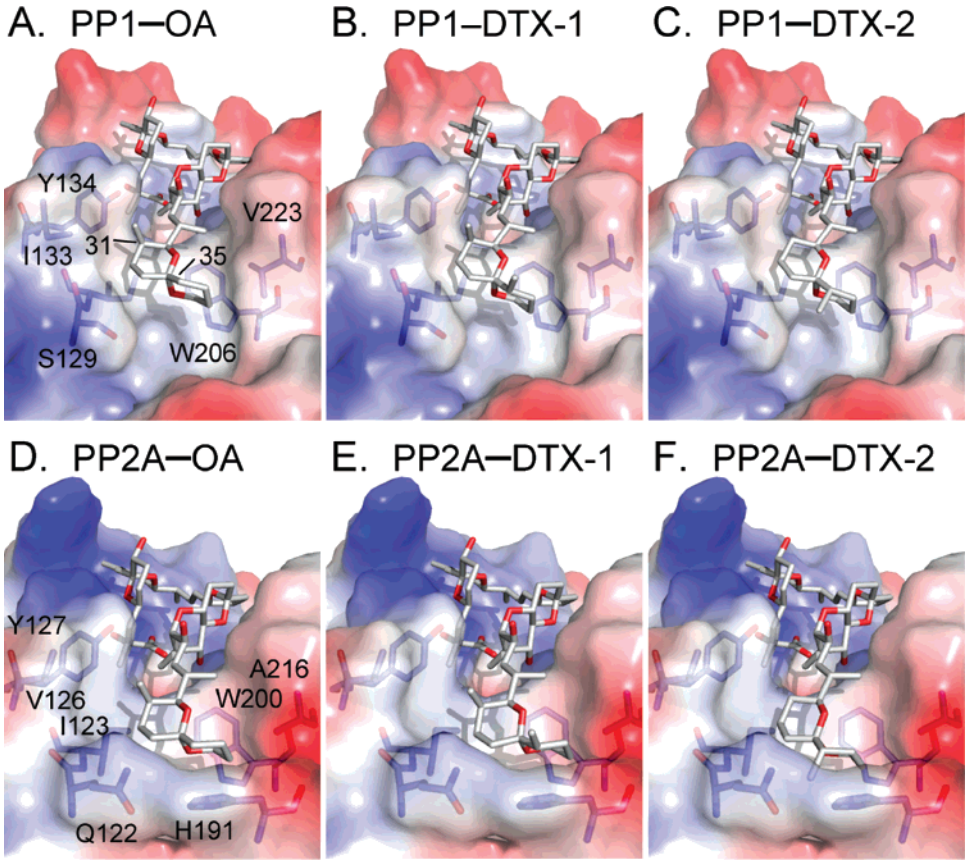

Figure 6. Molecular models of okadaic acid analogues docked to protein phosphatase-1 (PP1) and protein phosphatase 2A, on the basis of the crystal structures of okadaic acid bound to PP1 (PDB id 1JK7) and PP2A (*13*). (A) okadaic acid (**1**); (B) dinophysistoxin-1 (**2**); and (C) dinophysistoxin-2 (**3**) bound to PP1. (D) okadaic acid (**1**); (E) dinophysistoxin-1 (**2**); and (F) dinophysistoxin-2 (**3**) bound to PP2A. The electrostatic surface was generated using APBS (*41*), and the image was generated using PYMol (*42*).

at least for PP1, any difference in the inhibitory activity of DTX-2 (relative to OA and DTX-1) is more likely to be associated with the absence of the 31-methyl group and the consequent loss of the interaction of the 31-Me with Tyr134 in DTX-2 than to the presence of the axial 35-methyl group. An additional contribution may also arise from increased freedom of rotation about C-29−C-30 of unbound DTX-2, due to the loss of the steric interaction between the 31-Me and 29-Me groups, as increased loss of entropy upon binding a ligand imposes a significant binding energy penalty on receptor binding (*26*).

In contrast, the molecular docking of OA, DTX-1, and DTX-2 to PP2A shows a clear explanation for the reduced affinity for DTX-2 when compared with that for OA and DTX-1, and this is the direct result of the newly defined stereochemistry at position 35 (Figure 6D−F). Preliminary work indicated DTX-2 to have a similar inhibitory potency toward protein phosphatase-2A as okadaic acid and DTX-1 (*6*). In a subsequent detailed study, DTX-2 exhibited slightly reduced (ca. 0.6-fold) potency of PP2A inhibition and mouse toxicity relative to those of okadaic acid (*27*). The recent 3D structure of OA bound to human PP2A (*13*) provides a possible explanation for this observation because it appears that the axially orientated 35-methyl group of DTX-2 would make unfavorable close contacts with Gln122 and His191 (Figure 6F) at the end of the OA-binding pocket in addition to loss of the favorable contacts associated with the 31-Me group.

Studies of the biosynthesis of **1** and **2** and their esters in *Prorocentrum* spp. show that their 31- and 35-methyl groups both originate from the methyl groups of acetate units (*28*). Structural studies show that the 31-methyl groups of **1** and **2** are both installed with equatorial orientation and that **3** does not contain a 31-methyl group. The situation with respect to the 35-position is more complex: **1** does not contain a 35-methyl group, the 35-methyl group of **2** is installed with equatorial orientation, and the 35-methyl group of **3** is installed with axial orientation. Thus, the enzymes responsible for installing the 35-methyl group in DTX-1 operate with specificity opposite to those for installing the 35-methyl group of DTX-2. In algal blooms or contaminated seafood, typically either derivatives of OA (**1**) are the main component (e.g., *9*, *29*, *30*−*32*) or there is a significant co-occurrence of either **1** (no 35-methyl) and **2** (35*R*-methyl) (e.g., *9*, *33*, *34*−*37*), or of **1** (no 35-methyl) and **3** (35*S*-methyl) (e.g., *19*, *32*, *38*, *39*). The co-occurrence of substantial proportions of **2** (35*R*-methyl) and **3** (35*S*-methyl) together does not appear to have been reported. It seems likely, therefore, that organisms that biosynthesize OA analogues contain no 35-methylating enzyme, a 35-axial methylating enzyme, or a 35-equatorial methylating enzyme. If so, this would raise the possibility that **4** (35*R*-methyl but no 31-methyl) could potentially co-occur with **1** and **2** and that **5** (31-methyl and 35*S*-methyl) (i.e., 35*S*-**2**) could co-occur with **1** and **3**. Minor unidentified analogues of okadaic acid and DTXs have been reported by some workers (e.g., DTX-2b,c (*40*)), and some of these could correspond to **4**, **5**, or other isomers.

In summary, the results presented here show that DTX-1 (**2**) and DTX-2 (**3**) possess opposite stereochemistry at C-35. This finding has important implications for studies of the biosynthesis, biological activities, and synthesis of these compounds.

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structure of okadaic acid complexed with PP2A. This study was supported by Norwegian Research Council Grant 139593/140, by the BIOTOX project (partly funded by the European Commission, through 6th Framework Programme Contract No. 514074, topic Food Quality and Safety), and by the NZ Foundation for Research, Science and Technology International Investment Opportunities Fund (IIOF Contract Number C10X0406). The mussel material was donated by the Marine Institute, Ireland, (ASTOX project, funded by the National Development Plan (NDP) (ST/02/02 2003-2006) and the Marine Institute). The Norwegian Research Council (NFR) partially financed the Bruker Avance DRX 500 instrument and the full cost of the TXI cryo probe on the Bruker AV 600 instrument used in this study; The Faculty of Mathematics and Natural Sciences, University of Oslo, financed the Bruker AV 600 instrument; and the Bruker AV II 600 instrument and its TCI cryoprobe were fully financed by the NFR.

Supporting Information Available: NMR assignments for okadaic acid (**1**), DTX-1 (**2**), and DTX-2 (**3**) in $CDCl_3$ and CD_3CN; g-HSQC cross-sections through C-33 for **2** and **3**; a selected region of the HMBC NMR spectrum of DTX-1 (**2**) in $CDCl_3$ showing the strong correlation between H-9 (5.32 ppm) and the signal at 96.6 ppm that defines the latter as C-8; ^1H and ^{13}C NMR spectra of **1**, **2**, and **3** in CD_3OD, $CDCl_3$, and CD_3CN; and calculated interatomic distances for selected atoms in the docked **1**−PP1 complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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