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New Okadaic Acid Analogues from the Marine Sponge *Merriamum oxeato* and Their Effect on Mitosis

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Inhibitors of the G2 DNA damage checkpoint can selectively sensitize cancer cells with impaired p53 tumor suppressor activity to killing by DNA-damaging drugs or ionizing radiation and have been proposed as a promising therapeutic strategy. An extract from the Northeastern Pacific marine sponge *Merriamum oxeato* showed G2 checkpoint inhibitory activity, and fractionation identified the known dinoflagellate toxin dinophysistoxin 1 (**1**) and the two novel analogues 27-O-acetylokadaic acid (**2**) and 27-*O*-acetyldinophysistoxin 1 (**3**) as the active compounds. The mixture of **1**, **2**, and **3** was extremely potent at inhibiting the G2 checkpoint (IC_{50} = 1 ng/mL) and cellular protein Ser/Thr phosphatases (IC_{50} = 1 ng/mL), and it radiosensitized MCF-7 breast cancer cells expressing mutated p53 at all concentrations tested. However, the mixture of **1**, **2**, and **3** was also very toxic to cells not exposed to DNA damage (IC_{50} = 1 ng/mL), making these compounds poor candidates for therapeutic agents to augment the effectiveness of DNA-damaging therapies.

Cells normally respond to DNA damage by activating checkpoints that delay the transition from G1 to S and from G2 to M phases of the cell cycle while DNA is repaired. The checkpoints appear to serve a protective role by blocking replication of damaged DNA and segregation of damaged chromosomes. Most cancer cells have an inoperative G1 checkpoint due to p53 inactivation, and a functioning but impaired G2 checkpoint. Inhibitors of the G2 checkpoint can selectively sensitize cells with inactive p53 to killing by DNA-damaging drugs or ionizing radiation and have been proposed as a promising therapeutic strategy.[1]

Few G2 checkpoint inhibitors are known, and most of them are of limited clinical potential because they also interact with a broad range of other cellular processes.[2] Therefore, it is of considerable importance to find new and specific G2 checkpoint inhibitors that can be used to provide in vivo proof of principle and act as drug leads for this novel approach to cancer treatment. Toward this end, a cell-based assay developed in one of our laboratories has been used to screen marine invertebrate and terrestrial plant extracts for their ability to inhibit the G2 checkpoint.[3,4] Extracts of the Northeastern Pacific marine sponge *Merriamum oxeato* showed potent activity in the assay. Bioassay-guided fractionation of the extract identified the known dinoflagellate toxin dinophysistoxin 1 (**1**) and the two novel analogues 27-*O*-acetylokadaic acid (**2**) and 27-*O*-acetyl dinophysitoxin 1 (**3**) as the G2 checkpoint inhibitory components of the crude mixture. Details of the isolation and structure elucidation of **2** and **3** are reported below.

Results and Discussion

Specimens of *M. oxeato* were collected by hand using scuba off Nelson Island in Jervis Inlet, British Columbia. Freshly collected sponges were immediately immersed in MeOH and subsequently extracted repeatedly with fresh batches of MeOH over a period of several days. Concentration of the combined MeOH extracts in vacuo gave a bioactive oil that was first fractionated by solvent/solvent partitioning and subsequently by reversed-phase and Sephadex LH-20 chromatographies to provide an inseparable mixture of **1**, **2**, and **3** that exhibited G2 checkpoint inhibition at ng/mL concentrations. ^{1}H NMR analysis of the mixture of **1**, **2**, and **3** indicated that it was composed of three compounds related to okadaic acid.[5] In an attempt to simplify the separation of **1**, **2**, and **3**, a small portion of the mixture was converted to the corresponding mixture of methyl esters by treatment with $TMSCH_2N_2$ in MeOH/benzene.[6] However, recording the ^{1}H NMR spectrum of the crude reaction products in $CDCl_3$, in an attempt to verify that the methyl esters had been formed, resulted in complete decomposition. Therefore, the remaining sample of the mixture of **1**, **2**, and **3** was also treated with $TMSCH_2N_2$ in MeOH/benzene and the resulting reaction products were separated by reversed-phase HLPC into the pure methyl esters **4**, **5**, and **6**. NMR data for the methyl esters were recorded in C_6D_6 to avoid acid-catalyzed decomposition.

Methyl ester **4** gave a $[M + H]^+$ peak at *m/z* 833.5051 in the HRFABMS, appropriate for a molecular formula of $C_{46}H_{72}O_{13}$. Analysis of the 1D ^{1}H, HOHAHA, COSY, and HMQC NMR data collected for the ester **4**, combined with knowledge of the observed methylation patterns in the okadaic acid/dinophysistoxin family of polyether toxins, indicated that it was the methyl ester of dinophysistoxin 1 (**1**). Neither authentic dinophysistoxin 1 (**1**)[7] or its methyl ester **4** was available for comparison purposes, and there were no literature NMR data for either compound recorded in C_6D_6. Therefore, to confirm the structural assignment, authentic okadaic acid (**7**) (50 μg) was obtained from a commercial source and it was converted to the methyl ester **8** by treatment with $TMSCH_2N_2$.[6] Since there also were no literature NMR data for okadaic acid (**7**)[5] or its methyl ester **8** recorded in C_6D_6, the ^{1}H NMR data for the methyl

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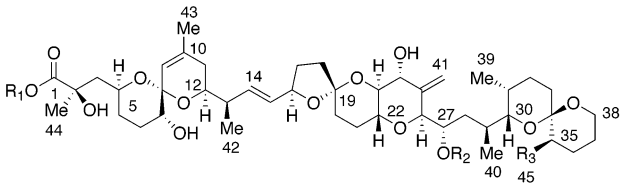
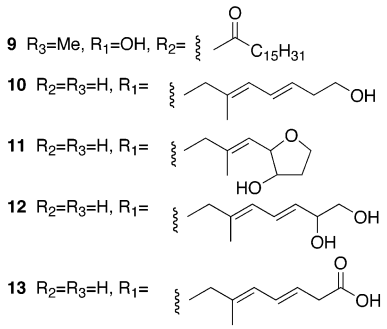
Table 1. ^{1}H NMR Data for Okadaic Acid Methyl Ester (**8**), Dinophysistoxin 1 Methyl Ester (**4**), 27-*O*-Acetylokadaic Acid Methyl Ester (**5**), and 27-*O*-Acetyldinophysistoxin 1 Methyl Ester (**6**) (all data recorded in C_6D_6 at 500 MHz)[a]

carbon no.	**8**	**4**	**5**	**6**
2	O*H*: 4.87 (s)	O*H*: 4.91 (s)	O*H*: 4.89 (s)	O*H*: 4.92 (s)
3		2.09 (m); 1.78 (m)	n.o.	2.09 (m); 1.82 (m)
4		4.15 (m)	4.12 (m)	4.14 (m)
5		1.80 (m); 1.31 (m)	n.o.	1.81 (m); 1.29 (m)
6		2.08 (m); 1.60 (m)	n.o.	1.87 (m); 1.57 (m)
7	3.46 (m)	3.46 (m)	3.42 (m)	3.41 (ddd, 4.1, 10.6, 10.6)
9	5.31 (s)	5.31 (bs)	5.30 (s)	5.30 (s)
11		1.51 (m); 1.33 (m)	1.54 (m); 1.50 (m)	1.50 (m); 1.34 (m)
12	3.81 (m)	3.81 (ddd, 4.8, 8.6, 8.6)	3.82 (ddd, 5.5, 9.3, 9.3)	3.81 (ddd, 5.5, 9.0, 9.0)
13		2.13 (m)	2.14 (m)	2.14 (m)
14	5.82 (dd, 8.7, 15.3)	5.82 (dd, 8.6, 15.2)	5.82 (dd, 8.8, 15.5)	5.82 (dd, 8.6, 15.2)
15	5.56 (dd, 7.8, 15.3)	5.56 (dd, 7.8, 15.2)	5.55 (7.8, 15.5)	5.54 (dd, 7.5, 15.2)
16	4.62 (m)	4.62 (ddd, 7.4, 7.6, 7.8)	4.62 (ddd, 7.6, 7.6, 7.8)	4.62 (ddd, 7.3, 7.5, 7.5)
17		2.03 (m); 1.49 (m)	1.98 (m); 1.52 (m)	2.13 (m); 1.51 (m)
18		n.o.	n.o.	n.o.
20		n.o.	n.o.	n.o.
21		1.95 (m); 1.52 (m)	2.08 (m); 1.80 (m)	2.02 (m); 1.94 (m)
22	3.48 (m)	3.49 (m)	4.13 (m)	4.12 ((m)
23	3.63 (dd, 9.4, 9.4)	3.64 (dd, 9.4, 9.8)	3.69 (dd, 9.8, 9.8)	3.69 (dd, 9.8, 9.8)
24	4.29 (m);	4.32 (m);	4.39 (m);	4.39 (m);
	O*H*: 2.75 (d, 5.5)	O*H*: 2.79 (d, 5.1)	O*H*: 2.83 (d, 4.7)	O*H*: 2.85 (d, 5.1)
26	3.96 (d, 9.2)	3.97 (d, 9.4)	4.21 (d, 9.4)	4.21 (d, 9.5)
27	4.16 (m); O*H*: 2.53 (s)	4.17 (m); O*H*: 2.57 (s)	5.85 (ddd, 2.0, 9.4, 9.4)	5.86 (ddd, 2.0, 9.5, 9.5)
28		1.45 (m); 1.04 (m)	1.52 (m); 1.06 (dd, 10,2, 10.2)	1.53 (m); 1.08 (dd, 9.6, 9.6)
29		2.33 (m)	1.81 (m)	1.82 (m)
30	3.44 (dd, 2.3, 10.2)	3.42 (dd, 2.3, 10.4)	3.32 (dd, 2.0, 10.3)	3.31 (dd, 2.0, 10.1)
31		1.82 (m)	n.o.	1.72 (m)
32		n.o.	n.o.	n.o.
33		n.o.	n.o.	n.o.
35	3.74 (m); 3.58 (m)	1.50 (m)	n.o.	1.43 (m)
36	1.11 (d, 7.0)	n.o.	n.o.	2.02 (m); 1.62 (m)
37	1.25 (d, 6.5)	1.61 (m), 1.34 (m)	1.49 (m); 1.38 (m)	1.54 (m); 1.26 (m)
38	5.64 (bs); 4.90 (bs)	3.68 (ddd, 2.4, 10.5, 10.5); 3.57 (dd, 4.7, 10.5)	3.50 (m); 3.62 (m)	3.52 (m); 3.54 (m)
39	0.97 (d, 6.8)	1.09 (d, 7.0)	0.98 (d, 6.6)	0.99 (d, 6.7)
40	1.37 (s)	1.22 (d, 6.5)	1.18 (d, 6.2)	1.16 (d, 6.4)
41	1.52 (s)	5.65 (bs); 4.90 (bs)	5.68 (bs); 4.91 (bs)	5.68 (bs); 4.90 (bs)
42	3.47 (s)	0.96 (d, 6.9)	0.97 (d, 6.7)	0.96 (d, 6.8)
43		1.36 (s)	1.36 (s)	1.35 (s)
44		1.52 (s)	1.53 (s)	1.53 (s)
45		1.00 (d, 6.7)		0.97 (d, 6.3)
46		3.47 (s)	3.49 (s)	3.48 (s)
47			1.79 (s)	1.76 (s)

[a] n.o. (not observed).



1 R_1=R_2=H, R_3=Me
2 R_1=R_3=H, R_2=Ac
3 R_1=H, R_2=Ac, R_3=Me
4 R_1=Me, R_2=H, R_3=Me
5 R_1=Me, R_2=Ac, R_3=H
6 R_1=Me, R_2=Ac, R_3=Me
7 R_1=R_2=R_3=H
8 R_1=Me, R_2=R_3=H



ester of the authentic okadaic acid **8** were recorded in C_6D_6, and diagnostic resonances were assigned as indicated in Table 1. Comparison of ^{1}H NMR spectra recorded for **4** and **8** revealed the presence of an additional methyl doublet (δ 1.00, J = 6.7 Hz, Me-45) in that of **4**. As expected, ^{13}C NMR analysis proved unsuccessful on such small amounts of material; however, inverse detection of many of the carbon resonances by a HMQC experiment confirmed the presence of seven appended methyl groups (see Table 2). Additionally, two protons that resonated at δ 3.68 and 3.57 in the ^{1}H NMR spectrum of **4** displayed a common correlation into a single carbon resonance at δ 59.8 (C-38) in the HMQC spectrum. A subsequent HOHAHA experiment provided connectivity between the protons at C-38 and a methyl group, which resonated at δ 1.00 in the ^{1}H NMR spectrum of **4**. Data gathered from a COSY experiment executed on **4** facilitated the connection of the additional methyl group to C-35. Comparison of the ^{13}C NMR chemical shifts for the methyl substituents at C-31 and C-35 in **4** to those of both known and novel (vide infra) okadaic acid analogues (see Table 3) permitted the assignment of the relative configuration at these methyl-bearing carbon centers.

27-*O*-Acetylokadaic acid methyl ester (**5**) gave a $[M + H]^+$ ion at m/z 861.5004 in the HRFABMS appropriate for a molecular formula of $C_{47}H_{72}O_{14}$, which differed from the molecular formula of the methyl ester of okadaic acid **8** by the addition of C_2H_2O. Analysis of the ^{1}H NMR spectrum

Table 2. ^{13}C NMR Data for Dinophysistoxin 1 Methyl Ester (**4**), 27-*O*-Acetylokadaic Acid Methyl Ester (**5**), and 27-*O*-Acetyldinophysistoxin 1 Methyl Ester (**6**) (from HMQC and HMBC data recorded at 500 MHz)[a]

carbon no.	**4**	**5**	**6**
1	n.o.	177.2	177.1
2	n.o.	75.9	75.4
3	n.o.	45.5	45.4
4	71.9	70.6	71.8
5	n.o.	n.o.	n.o.
6	n.o.	n.o.	n.o.
7	68.5	68.6	68.6
8	n.o.	n.o.	96.7
9	122.5	122.5	122.4
10	n.o.	138.0	138.0
11	n.o.	33.6	33.3
12	70.9	70.7	70.7
13	n.o.	42.7	42.9
14	135.6	135.4	135.7
15	131.1	131.3	131.2
16	79.1	78.9	78.9
17	n.o.	n.o.	31.0
18	n.o.	n.o.	n.o.
19	n.o.	101.4	n.o.
20	n.o.	n.o.	n.o.
21	n.o.	n.o.	26.5
22	69.7	70.1	70.1
23	77.1	77.2	77.2
24	71.2	71.4	71.5
25	n.o.	n.o.	145.1
26	85.1	83.4	83.5
27	65.0	67.3	67.4
28	n.o.	31.7	33.9
29	n.o.	34.2	34.0
30	75.0	74.4	74.3
31	n.o.	28.0	28.1
32	n.o.	26.9	26.8
33	n.o.	n.o.	n.o.
34	n.o.	n.o.	98.2
35	n.o.	n.o.	39.6
36	n.o.	n.o.	37.3
37	n.o.	n.o.	n.o.
38	59.8	60.1	59.8
39	10.9	10.5	10.5
40	16.3	16.2	16.1
41	112.2	112.3	112.3
42	15.9	15.9	16.2
43	22.5	22.4	22.4
44	27.4	27.2	27.2
45	17.0		16.2
46	51.8	51.7	51.7
47		170.1	170.2
48		20.1	20.2

[a] n.o. (not observed).

Table 3. ^{13}C NMR Chemical Shifts for C-39 and C-45 in Okadaic Acid (**7**),[8] Dinophysistoxin 1 (**1**),[8] Dinophysistoxin 1 Methyl Ester (**4**), 27-*O*-Acetylokadaic Acid Methyl Ester (**5**), and 27-*O*-Acetyldinophysistoxin 1 Methyl Ester (**6**)

carbon no.	okadaic acid (**7**)[a]	dinophysistoxin 1 (**1**)[a]	**4**[b]	**5**[b]	**6**[b]
C-39	10.7	10.7	10.9	10.5	10.5
C-45		16.8	17.0		16.2

[a] Recorded at 75.5 or 125.8 MHz in $CDCl_3$. [b] From HMQC and/or HMBC data recorded at 500 MHz in C_6D_6.

obtained for **5** revealed that this substance was closely related to **8** (Table 1). A downfield shift of the H-27 resonance to δ 5.85 ppm in the spectrum of **5**, relative to its position at δ 4.16 ppm in the spectrum of okadaic acid methyl ester **8**, suggested an acyl shift. The spectrum of **5** also contained a new methyl resonance at δ 1.79 and was missing the 27-O*H* resonance at δ 2.53 ppm compared to the spectrum of **8**. Taken together, the ^1H NMR and FABMS data indicated that **5** was the 27-*O*-acetyl derivative of okadaic acid methyl ester **8**. The small sample size precluded direct detection of a ^{13}C NMR spectrum for **5**. However, many of the carbon resonances could be assigned from the HMQC and HMBC data as indicated in Table 2. A HMBC correlation was observed between the methyl resonance at δ 1.79 and a carbonyl resonance at δ 170.2, consistent with the presence of an acetyl residue, and a HMQC correlation observed between the H-27 resonance at δ 5.85 and a carbon resonance at δ 67.3 showed that the C-27 resonance in **5** was deshielded by 2.3 ppm relative to its counterpart in **4**, in agreement with attachment of an acetoxy residue to C-27. Data from a COSY and a series of 1D TOCSY experiments facilitated the ^1H NMR assignments for **5** given in Table 1.

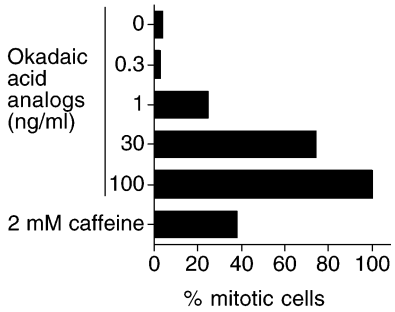


Figure 1. G2 checkpoint inhibition by the mixture of **1**, **2**, and **3** and by caffeine.

27-*O*-Acetyldinophysistoxin 1 methyl ester (**6**) gave a $[M + H]^+$ ion at *m/z* 875.5151 in the HRFABMS appropriate for a molecular formula of $C_{48}H_{74}O_{14}$, which differed from the formula of 27-*O*-acetylokadaic acid methyl ester (**5**) by the addition of CH_2. Analysis of the 1D ^1H, 1D TOCSY, COSY, HMQC, and HMBC data obtained for **6** confirmed that it was closely related to **4** and **5**. The most obvious difference between the ^1H NMR spectra of **5** and **6** was the presence of one additional methyl resonance (δ 0.97, d, J = 6.3 Hz: Me-45) in the ^1H NMR spectrum of **6** (Table 1). Once again, the H-27 resonance had a chemical shift of δ 5.86 ppm in the ^1H NMR spectrum of **6**, and there was a methyl resonance at δ 1.76 that showed a HMBC correlation to an ester carbonyl resonance at δ 170.2 ppm, in agreement with the presence of a C-27 acetoxy substituent. The new methyl resonance (δ 0.97) in the ^1H NMR spectrum of **6** showed a HMBC correlation to the C-34 ketal resonance at δ 98.2 ppm. Further analysis of COSY spectral data and a series of 1D TOCSY experiments permitted the connection of the additional methyl group to C-35, suggesting that **6** was simply the 27-*O*-acetyl derivative of dinophysistoxin 1 methyl ester (**5**). All of the 1D and 2D NMR data obtained for **6** were consistent with this assignment.

It has been reported that some organisms that feed on toxic dinoflagellates may in fact be capable of detoxifying okadaic acid and the dinophysistoxins through chemical transformations. The scallop *Patinopectin yessoensis*, when reared on the dinophysistoxin 1 (**1**)-producing dinoflagellate *Dinophysis fortii*, rapidly converts **1** to 7-*O*-acyl dinophysistoxin 1 (**9**).[9] Additionally, the diatom *Thalassiosira weissflogii* is capable of transforming the toxic diol ester of okadaic acid **10**, produced by *Prorocentrum lima*, to the more polar oxidation products **11**−**13**.[10] Okadaic acid (**7**), or analogues thereof, were not detected in any of the extracts from the assemblage of sponges collected at similar depths and location to *M. oxeato*. Thus, the accumulation of acetylated dinoflagellate toxins (i.e., **2** and **3**) in the sponge tissue raises interesting questions. Since there are no reports in the literature of dinoflagellate toxins acety-

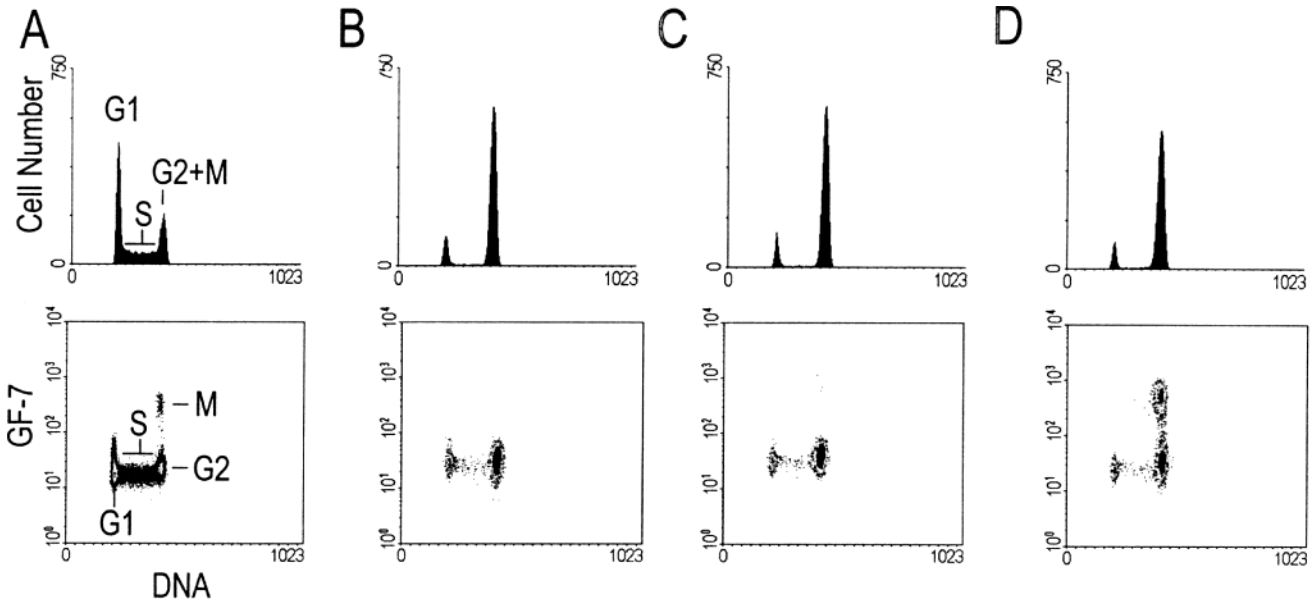


Figure 2. Flow cytometric analysis of G2 checkpoint inhibition and mitotic arrest induced by the mixture of **1**, **2**, and **3**. Cellular DNA content was determined by propidium iodide staining, and mitotic cells were determined using the GF-7 antibody. Panel A: untreated cycling cells. Panel B: cells 16 h after irradiation. Panel C: cells 20 h after irradiation. Panel D: cells treated with the mixture of **1**, **2**, and **3** between 16 and 20 h after irradiation. The upper row shows histograms of the DNA profiles, and the lower row shows the mitotic GF-7 fluorescence plotted against the DNA signal. The regions of the plot corresponding to cells in G1, S, G2, and M are indicated in panel A.

lated at C_{27}-OH, and this hydroxyl function is reportedly the least nucleophilic in the molecule,[11] it is possible that the sponge *M. oxeato* is in fact acetylating the parent toxins. However, substantiation of this statement requires the identification of the DSP-producing dinoflagellate[12] and analysis of the toxins produced by this alga.

The mixture of **1**, **2**, and **3** was assayed for G2 checkpoint inhibition by exposing G2-arrested cells to different concentrations of the mixture for 4 h and then determining the number of cells that had escaped G2 arrest and entered mitosis.[3] As shown in Figure 1, the mixture exhibited significant G2 checkpoint inhibition at 1 ng/mL and caused complete inhibition of the G2 checkpoint at 100 ng/mL. Caffeine, the reference standard G2 checkpoint inhibitor, caused 37% G2 checkpoint inhibition at its optimal concentration of 2 mM (400 μg/mL). The mixture of okadaic acid analogues **1**, **2**, and **3** represents the only compounds we have found to date that are able to induce 100% of G2-arrested cells to enter mitosis.

G2 checkpoint inhibition by the okadaic acid analogues was examined in more detail using a flow cytometry procedure. The cells were labeled with the DNA dye propidium iodide and with the GF-7 antibody to mark mitotic cells and analyzed by flow cytometry to determine the proportion of cells in the different phases of the cell cycle. As shown in Figure 2, nonirradiated cycling cells contained 40% G1 phase cells, 30% S phase cells, 27% G2 cells, and 3% M cells (panel A). Exposure of cells to 6.5 Gy ionizing radiation induced G2 arrest, with 90% cells arrested in G2 phase 16 h after irradiation (panel B). Cells harvested 20 h after irradiation remained arrested in G2 phase (panel C). However, when cells were treated with the mixture of **1**, **2**, and **3** (25 ng/mL) between 16 and 20 h after irradiation, 50% of the cells escaped G2 arrest as measured by their accumulation in M phase (panel D), demonstrating that the okadaic acid analogues abrogated the G2 checkpoint. Moreover, no increase in the number of cells in G1 and S was observed, showing that in addition to overcoming G2 arrest, the okadaic acid analogues also caused the cells to block in M phase.

Okadaic acid is a potent inhibitor of protein (Ser/Thr) phosphatases. We next compared protein phosphatase inhibition by okadaic acid and the okadaic acid analogues. As shown in Figure 3, the okadaic acid analogues were also potent inhibitors of cellular protein (Ser/Thr) phosphatases, with an IC_{50} of 0.3 ng/mL, very close to that of okadaic acid (IC_{50} = 0.6 ng/mL).

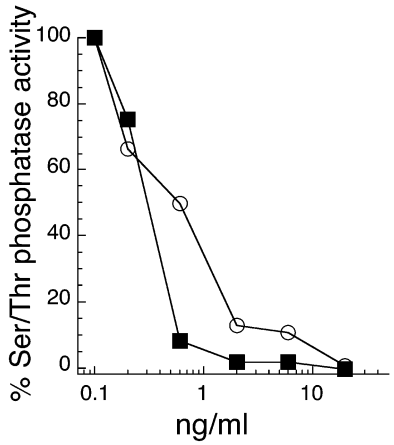


Figure 3. Inhibition of cellular Ser/Thr phosphatases by the mixture of **1**, **2**, and **3** (■) and by okadaic acid (○).

G2 checkpoint inhibitors can often potentiate cell killing by DNA-damaging agents, presumably by forcing cells to undergo mitosis before completion of DNA repair. To determine whether this is the case for the okadaic acid analogues, cells were treated with different concentrations of the mixture of **1**, **2**, and **3** and were exposed immediately afterward to 0 or 6.5 Gy ionizing radiation. The drugs were washed away after 20 h, and the cells were allowed to grow for several days. Cell proliferation was then determined. As shown in Figure 4, the okadaic acid analogues were very potent cytotoxic agents on their own, with an IC_{50} of 1 ng/mL. They also potentiated killing by ionizing radiation at all concentrations tested.

To investigate how the okadaic acid analogues overcome G2 arrest, we next examined their effects on cellular protein kinase activity using an in-gel kinase assay. These

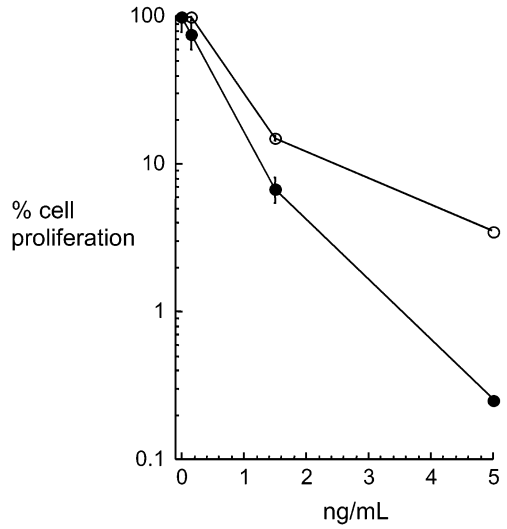


Figure 4. Inhibition of cell proliferation by the okadaic acid analogues. Cells were treated with different concentrations of the mixture of **1**, **2**, and **3** and irradiated (●) or not (○).

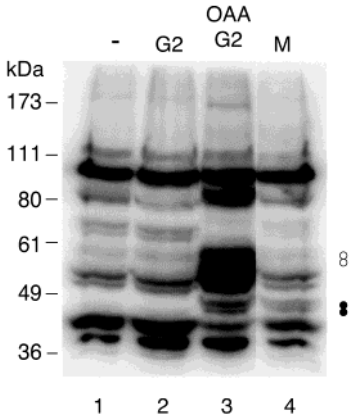


Figure 5. Effect of the mixture of **1**, **2**, and **3** on cellular protein kinases. Cycling cells (−), G2 arrested cells (G2), G2 arrested cells treated with okadaic acid analogues (OAA G2), and cells arrested in mitosis (M) were subjected to in-gel kinase assay. Protein kinases activated at mitosis (●) and protein kinases activated by stress and apoptosis-inducing stimuli (○) are shown to the right and migration of protein standards, in kDa, is shown to the left.

assays are based on the observation that many protein kinases can be renatured after separation by gel electrophoresis under denaturing conditions. By polymerizing an appropriate substrate within the gel and incubating with (γ^{32}P)ATP, the activity of several protein kinases can be detected. Extracts prepared from cycling cells or from G2-arrested cells showed identical patterns of protein kinase activity (Figure 5, lanes 1 and 2), while mitotic cells showed a different pattern characterized by the presence of two active kinases around 45 kDa (Figure 5, lane 4, indicated by filled dots).[13] Extracts prepared from G2-arrested cells treated with the okadaic acid analogues for 4 h displayed these two bands (Figure 5, lane 3), showing that they cause the activation of protein kinases normally active at mitosis. However, it is clear that the okadaic acid analogues also activated protein kinases that are not normally active in cycling, G2-arrested, or mitotic cells. In-gel kinase assays have also been used to analyze proteins activated by stress and apoptosis-inducing stimuli such as heat shock, sodium arsenite, staurosporine, and okadaic acid.[14] These assays show a characteristic doublet of kinases at 61 and 63 kDa that are activated only by stress stimuli and have been identified as Mst1 and Mst2, respectively.[14] These kinases were also activated prominently by the okadaic acid analogues (Figure 5, indicated by open dots) and by staurosporine and sodium arsenite (results not shown). This result indicates that the okadaic acid analogues activate both entry into mitosis and a stress or apoptotic response in G2-arrested cells.

This study has identified new okadaic acid analogues that are extremely potent and efficacious inhibitors of the G2 checkpoint, are potent inhibitors of cellular Ser/Thr phosphatases, and can act as radiosensitizing agents. It is conceivable that checkpoint inhibition and induction of apoptosis might both contribute to radiosensitization by these okadaic acid analogues. The high toxicity of the okadaic acid analogues to cells that have not been exposed to DNA damage would seem to preclude them as candidates for therapeutic agents to augment the effectiveness of DNA-damaging therapies. Nevertheless, links between protein phosphatases and the etiology or control of human diseases including cancer are emerging, and some protein phosphatase inhibitors show antitumor activity, prompting the recent proposal of inhibition of Ser/Thr phosphatases as a therapeutic strategy.[15,16] The identification of additional small molecule inhibitors of this class of enzymes is an important step in the testing of this therapeutic hypothesis.

Experimental Section

Samples of *Merriamum oxeato* (1 kg wet weight) were collected off the northwest and southwest points of Nelson Island, in Jervis Inlet, British Columbia, in September of 1999 and January of 2000 using scuba at depths of 25−30 m. A voucher sample of the sponge has been deposited at the University of Amsterdam. Freshly collected sponge was immediately immersed in MeOH and transported back to the University of British Columbia. The MeOH was decanted after 24 h, and the sponge was immersed in fresh MeOH. After the sponge had been extracted seven times (over 7 days) with MeOH, the combined extracts were concentrated to provide a green gum. The green gum was dissolved in H_2O (500 mL) and EtOAc (500 mL), and the phases were separated. The aqueous phase was washed with EtOAc (10 × 250 mL), and the combined organic phases were concentrated in vacuo to provide a brown residue. The residue was dissolved in MeOH/H_2O (9:1, 100 mL) and extracted with hexane (3 × 50 mL). The MeOH/H_2O phase was then concentrated in vacuo to provide a green residue, and the combined hexane phases were concentrated in vacuo to provide an orange oil.

The green bioactive residue was placed on a short plug of reversed-phase silica gel (10 g), and the most polar compounds in the mixture were eluted with MeOH/H_2O (1:5, 10 mL). Compounds of decreasing polarity were then eluted with MeOH/H_2O (2:5, 10 mL, fraction A), (4:5, 10 mL, fraction B), (1:1, 10 mL, fraction C), (5:3, 10 mL, fraction D), (5:1, 10 mL, fraction E), (5:0, 10 mL, fraction F); MeOH/EtOAc (5:1, 10 mL, fraction G); and EtOAc (10 mL, fraction H). A biological assay of the individual fractions indicated fractions E and F contained compounds that inhibit the G2 checkpoint. Combination and concentration of fractions E and F provided a brown residue (25 mg), which was placed on a Sephadex LH-20 column and eluted with MeOH. Combination and concentration of all active fractions provided a beige residue (5 mg), which was placed on a Sephadex LH-20 column and eluted with EtOAc/MeOH/H_2O (25:5:1) to provide an inseparable mixture of compounds, which included dinophysistoxin 1 (**1**), 27-*O*-acetylokadaic acid (**2**), and 27-*O*-acetyldinophysistoxin 1 (**3**) (total weight 1.5 mg, ^{1}H NMR ratio 2:3:3), as a white solid.

Preparation of Dinophysistoxin 1 Methyl Ester (4), 27-*O*-Acetylokadaic Acid Methyl Ester (5), and 27-*O*-Acetyldinophysistoxin 1 Methyl Ester (6). To a stirred solution of the three okadaic acid analogues **1**, **2**, and **3** (1.0 mg, 0.001 mmol) in dry MeOH/benzene (1:7, 1.6 mL) at room tempera-

ture was added a solution of (trimethylsilyl)diazomethane (2.0 M in hexane, 20 μL, 0.01 mmol). After 2 h, the reaction mixture was concentrated under reduced pressure. Purification of the crude methyl esters by reversed-phase HPLC (analytical column, 1 mL/min, monitor at 204 nm, 78:20 MeOH/H_2O) provided 0.2 mg (0.00002% wet weight) of dinophysistoxin 1 methyl ester (**4**) as a white solid (retention time 14 min), 0.3 mg (0.00003% wet weight) of 27-*O*-acetylokadaic acid methyl ester (**5**) as a white solid (retention time 20.4 min), and 0.3 mg (0.00003% wet weight) of 27-*O*-acetyldinophysistoxin 1 methyl ester (**6**) as a white solid (retention time 26.5 min).

Dinophysistoxin 1 Methyl Ester (4): (RP-TLC $R_f = 0.6$, 8:1 MeOH/H_2O); ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; HRFABMS $[M + H]^+$ *m/z* 833.5053, exact mass calcd for $C_{46}H_{73}O_{13}$ 833.5051.

27-*O*-Acetylokadaic acid methyl ester (5): (RP-TLC $R_f = 0.55$, 8:1 MeOH/H_2O); ^{1}H NMR see Table 1; ^{13}C NMR, see Table 2; HRFABMS $[M + H]^+$ *m/z* 861.5004, exact mass calcd for $C_{47}H_{73}O_{14}$ 861.5000.

27-*O*-Acetyldinophysistoxin 1 methyl ester (6): (RP-TLC $R_f = 0.5$, 8:1 MeOH/H_2O); ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; HRFABMS $[M + H]^+$ *m/z* 875.5151, exact mass calcd for $C_{48}H_{75}O_{14}$ 875.5157.

G2 Checkpoint Inhibition Assay. Human mammary tumor MCF-7 cells expressing a dominant negative mutant p53 gene were grown as monolayers. G2 arrest was induced by exposing cycling cells to 6.5 Gy ionizing radiation from a ^{60}Co source (Gammacell 200, Atomic Energy Commission of Canada) at a dose rate of 1.2 Gy/min with a uniform irradiation field. G2 checkpoint inhibition was determined using an ELISA.[3]

Flow Cytometry. Cells were prepared for flow cytometry as described previously.[4a] Briefly, cells were harvested, fixed in ice-cold 70% ethanol, and permeabilized with detergent. They were then incubated with GF-7 monoclonal antibody[17] and with FITC-conjugated anti-mouse antibody, followed by digestion with RNase I and staining of DNA with propidium iodide. The cells were analyzed with a Becton-Dickinson FACScan using gating to eliminate cell debris and clumps.

Ser/Thr Phosphatase Inhibition Assay. A whole cell extract containing protein phosphatases was prepared by lysing MCF-7 cells in ice-cold 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% Triton X-100, 0.1 mM EDTA, 10% glycerol, 0.1 mM PMSF, 50 μg/mL aprotinin, and 50 μg/mL leupeptin. The extract was desalted on a Sephadex G-25 spin column to remove free phosphate, and aliquots of extract were stored at −70 °C. Protein phosphatase activity was determined using peptide RRAT(PO_3H_2)VA-amide as the substrate and a molybdate-malachite green colorimetric assay according to Technical Bulletin No. 218 (Promega, Madison, WI).

Cell Proliferation Assay. Cells were seeded at 1000 cells per well in 96-well plates, grown overnight, and treated with different concentrations of the mixture of **1**, **2**, and **3**. Immediately after addition of the mixture of okadaic acid analogues, the cells were irradiated with 0 or 6.5 Gy. After 24 h, the mixture of okadaic acid analogues was removed and the cells were allowed to grow in fresh medium until those not treated with drug approached confluency, typically between 3 days for nonirradiated cells and 7 days for irradiated cells. Cell proliferation was measured using the MTT assay as follows: 25 μL of a 5 mg/mL solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in phosphate-buffered saline was added to cells in the presence of 100 μL of cell culture medium. After 2 h incubation at 37 °C, 100 μL of a solution of 20% sodium dodecyl sulfate dissolved in dimethylformamide/water (1:1, pH 4.7) was added, and after overnight incubation, absorbance at 570 nm was measured.

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