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4. Proliferative assays were performed with 3×10^4 clonal responder cells per well in round-bottomed microtiter plates (Costar). The E rosette–negative PBMC population (autologous or allogeneic, both irradiated with 5000 rad) at 3×10^5 cells per well served as APC's. Antigen concentrations were 10 μg/ml. After 24 hours of culture in vitro at 37°C in a humidified atmosphere of 7 percent CO_2 in air, the wells were individually exposed to 1 μCi of [^{3}H]thymidine and washed 18 hours later. [^{3}H]Thymidine uptake was measured in a Packard liquid scintillation spectrometer. Antigen/MHC–induced proliferation of RW17C (5593 count/min) was inhibited by antisera to Ia but not to HLA (*5*) (anti-Ia: 952 count/min; anti-HLA: 5722 count/min). In addition, anti-Ti_{4A} at 10 μg/ml inhibited RW17C when stimulated with the antigen/MHC (624 count/min) but had no influence on any other clone. In contrast, anti-$T3_C$ blocked antigen/MHC–induced proliferation of all the T cell clones tested. In the presence of the last two monoclonals, the respective clone's proliferative responses to IL-2 were markedly enhanced, consistent with previous observations (*8*) and excluding nonspecific antibody effects. In addition, anti-T4 (10 μg/ml) inhibited clonal proliferation to antigen/MHC (418 count/min). Induction of IgG secretion was assessed by incubating 5×10^4 E rosette–negative, T cell depleted [lysis with anti-T4, anti-T8, and rabbit complement (*13*)] autologous or allogeneic PBMC per well with 2.5×10^4 cloned RW17C cells and antigens (10 μg/ml) or media for 7 days at 37°C in a humidified atmosphere of air and 7 percent CO_2 in triplicate round-bottomed microtiter plates. Subsequently, IgG was assayed in supernatants by means of a solid phase radioimmunoassay (*13*). Standard deviations in all experiments were < 15 percent. Results are representative of seven experiments performed. Values given for proliferation and IgG concentration represent means of triplicate cultures.
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Bromine Residue at Hydrophilic Region Influences Biological Activity of Aplysiatoxin, a Tumor Promoter

Abstract. *Aplysiatoxin and debromoaplysiatoxin, which are isolated from the seaweed,* Lyngbya gracilis, *differ in their chemical structure only by the presence or absence of a bromine residue in the hydrophilic region. The function and the structure-activity relation of the hydrophilic region are not known. Aplysiatoxin increased malignant transformation, stimulated DNA synthesis, and inhibited the binding of phorbol-12,13-dibutyrate and epidermal growth factor to cell receptors. Debromoaplysiatoxin inhibited the binding of these two substances as strongly as aplysiatoxin but did not increase malignant transformation or stimulate DNA synthesis. These results indicate that a slight change in the chemical structure of the hydrophilic region of aplysiatoxin affects its abilities to increase cell transformation and stimulate DNA synthesis and that the abilities of the tumor promoters to inhibit the binding of phorbol-12,13-dibutyrate and epidermal growth factor are dissociable from their abilities to increase cell transformation and stimulate DNA synthesis under some circumstances.*

The presence of many kinds of carcinogens, initiators, and tumor promoters in the human environment has been widely recognized. Sugimura *et al.* (*1, 2*) screened environmental substances to seek new tumor promoters, first by testing the irritancy of the substance on mouse ear, and second by testing the substance for induction of ornithine decarboxylase (ODC) activity in mouse skin. Some compounds that showed high activity in these two tests did not promote tumors when the compounds were painted on mouse skin. The most interesting of these compounds is a polyacetate derivative, debromoaplysiatoxin, the chemical structure of which differs from that of aplysiatoxin only by a lack of bromine residue at the hydrophilic region. Both aplysiatoxin and debromoaplysiatoxin were isolated from a seaweed, *Lyngbya gracilis*. Debromoaplysiatoxin did not improve the adhesion of human promyelocytic leukemia cells (HL-60) to the substrate, whereas aplysiatoxin showed high activity in all of the tests described above, including the test of promoting skin tumors in vivo.

The sequential process and the mechanisms by which diverse effects are induced by the skin tumor–promoting agents are not well understood. The difference between the effects of aplysiatoxin and debromoaplysiatoxin may shed light on the sequential process of promoter action and the functional role of the hydrophilic region of promoters. However, the effects of promoters vary markedly depending on the type and kind of cells and the genetic and physiological condition of the cells (*3*). It is difficult to link diverse observations obtained with different systems or cells. Exclusive use of transformable cultured

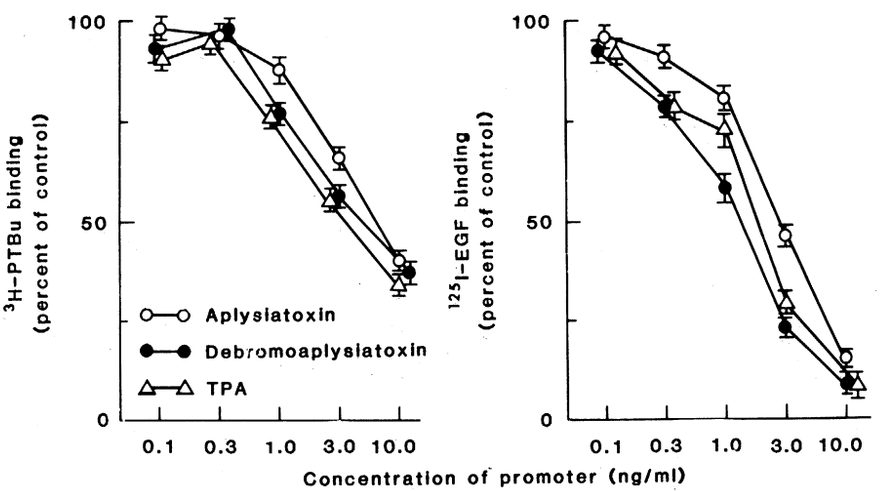

Fig. 1. Inhibition of PDBu and EGF binding by tumor promoter. Cells (2×10^6 per dish) were incubated with 2 ml of Eagle's minimum essential medium (MEM) containing ovalbumin (2 mg/ml), various concentrations of tumor promoter, and 0.2 ng of ^{125}I-labeled EGF (120 mCi/mg; Collaborative Research) for 45 minutes or 5.8 ng of ^{3}H-labeled PDBu (35 mCi/mg; New England Nuclear) for 30 minutes. The cells were then washed three times with MEM containing ovalbumin (2 mg/ml) and lysed with 0.1*N* NaOH, and cell-bound radioactivity was determined.

cells as the model system for carcinogenesis in vivo is advantageous for elucidating the cellular and molecular mechanisms of action of tumor promoters. We now report the results obtained by using a promoter-sensitive and transformable clone, mouse BALB/3T3 A31-1-1 cells (*4, 5*). Different biological responses were induced by aplysiatoxin and debromoaplysiatoxin.

BALB/3T3 A31-1-1 cells (*4*) were used throughout the experiments. In the first series of experiments, aplysiatoxin and debromoaplysiatoxin were examined to determine whether or not they increase 3-methylcholanthrene (MCA)-initiated malignant transformation as judged by comparison with phorbol-12,13-didecanoate (PDD), which is a potent tumor promoter. Alone, MCA (1 μg/ml) induced very low transformation frequencies under the conditions used, although a higher concentration of MCA induced transformation in a dose-dependent manner (*6*). Treatment with aplysiatoxin, at the concentrations of 3 and 10 ng/ml for 2 weeks, increased the number of transformation foci in MCA-treated cells in comparison with cells that were not treated with aplysiatoxin (Table 1). The potential of aplysiatoxin for increasing cell transformation was equivalent to that of PDD. In contrast, debromoaplysiatoxin at concentrations up to 10 ng/ml did not increase transformation frequency. Treatment with promoters alone did not induce cell transformation. One transformed focus was observed in one of 11 cultures exposed to aplysiatoxin (10 ng/ml); the significance of this observation is not known. The reduction of cloning efficiencies was slight, even when the promoters were added to cultures 24 hours after the cells were plated (Table 1). In the transformation experiments, the tumor promoters were added to cultures 7 days after the cells were plated, a time when all cultures had reached the confluent state. Thus it is likely that the cytotoxicity of promoters is negligible in the calculation of transformation frequencies.

Tumor promoters of the phorbol ester type modify or inhibit the binding of epidermal growth factor (EGF) and phorbol-12,13-dibutyrate (PDBu) to cell receptors (*5, 7–9*). Aplysiatoxin, debromoaplysiatoxin, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), used as control tumor promoters, inhibited almost equally the binding of ^{125}I-labeled EGF and ^{3}H-labeled PDBu to the cells (Fig. 1).

Induction of DNA synthesis in arrested cells was examined as a late response

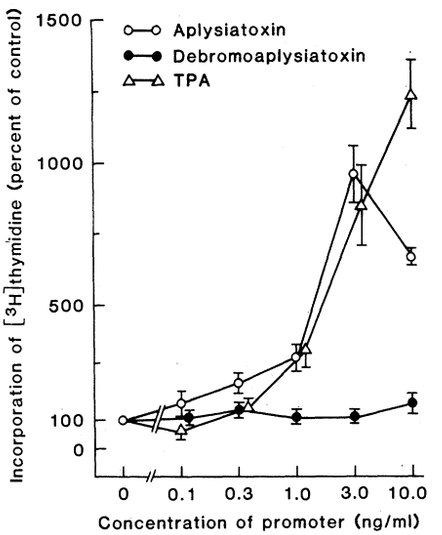

Fig. 2. Induction by tumor promoters of DNA synthesis in arrested cells. The confluent and density-inhibited cultures, prepared earlier by plating 3×10^5 cells and incubating for 1 week, were incubated for 24 hours with 5 ml of conditioned medium containing 20 ng of [^{3}H]thymidine (100 mCi/mg; Amersham) and various concentrations of tumor promoters. The cells were then scraped into phosphate-buffered saline, trapped on glass filters, and washed twice with cold 5 percent trichloroacetic acid. The filters were washed twice with cold water, dried, immersed in toluene and diphenyloxazole, and measured for radioactivity with a liquid scintillation counter. Conditioned medium was prepared by exposing fresh medium containing 10 percent fetal bovine serum to confluent cultures of A31-1-1 cells for at least 3 days.

of cells to tumor promoters (*5, 10*) (Fig. 2). Treatment with aplysiatoxin and TPA at concentrations ranging between 1.0 and 10.0 ng/ml produced marked induction of DNA synthesis in arrested cells, whereas debromoaplysiatoxin at concentrations up to 10.0 ng/ml did not.

The findings that aplysiatoxin increased cell transformation in MCA-treated cells but debromoaplysiatoxin did not parallel the finding that aplysiatoxin, when painted on mouse skin, greatly increased tumor formation, whereas debromoaplysiatoxin did so only slightly. However, aplysiatoxin, debromoaplysiatoxin, and TPA equally inhibited EGF and PDBu binding to cells. This finding suggests that these three compounds may act on the same receptor system in cells. Horowitz *et al.* (*11*) reported that debromoaplysiatoxin was about ten times weaker than aplysiatoxin in inhibiting EGF and PDBu binding to mouse 10T1/2 cells. They reported effective doses of aplysiatoxin and TPA for inhibiting receptor binding that were identical to our values, suggesting that experimental conditions were similar in the two laboratories. We confirmed the reproducibility of our results with a different batch of aplysiatoxin and debromoaplysiatoxin. The cause of the discrepancy between our results and those of Horowitz *et al.* on the relative ability of debromoaplysiatoxin to inhibit receptor binding may be due to the differences in the lipid milieu of phorbol ester receptors in the two cell types. The potential of phorbol ester derivatives for inhibiting

Table 1. Effects of aplysiatoxin and debromoaplysiatoxin on transformation frequencies in BALB/3T3 cells initially transformed with 3-methylcholanthrene (MCA). Cells (10^4) were plated in plastic dishes (diameter, 60 mm) containing 5 ml of Eagle's minimum essential medium supplemented with 10 percent fetal bovine serum. After 24 hours the cells were treated with MCA at the concentration of 1 μg/ml for 72 hours. The cultures were subsequently incubated with MCA-free medium for 4 days, with medium containing aplysiatoxin, debromoaplysiatoxin, or PDD for 2 weeks, and then with promoter-free medium for 2 weeks. The cells were fixed with methanol and stained (Giemsa) 5 weeks after they were plated. The transformed foci were scored by the method of Kakunaga (*6, 13*). Cytotoxic effects of promoters were measured by the reduction of cloning efficiency when 100 cells were plated, exposed to the medium containing various concentrations of tumor promoter for 1 week beginning 24 hours after cell plating, and fixed. All chemicals were dissolved in dimethyl sulfoxide (at a final concentration of 0.5 percent) and added to the culture medium. The plating efficiency is given as the average value of ten dishes in each group. The number of transformed foci per dish is the mean ± standard error for the number of dishes given in parentheses.

Tumor promoter	Dose (ng/ml)	Plating efficiency (%)	Transformed foci per dish	
			Untreated	Treated with MCA
None	0	100	0 (8)	0.78 ± 0.28 (9)
PDD	10.0	92.7	0 (11)	3.56 ± 0.23 (10)
Aplysiatoxin	0.3	85.8	0 (10)	0.82 ± 0.24 (10)
	1.0	91.7	0 (10)	0.98 ± 0.34 (10)
	3.0	89.3	0 (10)	1.74 ± 0.35 (11)
Debromo-aplysiatoxin	10.0	80.7	0.09 ± 0.09 (11)	4.05 ± 0.10 (11)
	0.3	96.0	0 (10)	0.52 ± 0.28 (10)
	1.0	89.0	0 (9)	0.90 ± 0.40 (10)
	3.0	89.1	0 (11)	0.82 ± 0.27 (11)
	10.0	85.5	0 (11)	0.64 ± 0.33 (11)

EGF and PDBu binding has been reported to correlate generally with their activity in promoting mouse skin carcinogenesis (*9, 12*). However, even though debromoaplysiatoxin did not increase MCA-initiated cell transformation, this polyacetate derivative markedly inhibited the binding of EGF and PDBu to cell receptors. There was an apparent dissociation between the activity of debromoaplysiatoxin in binding or modulating receptors and its activity in increasing MCA-induced cell transformation. We earlier reported a similar observation, namely that dihydroteleocidin B was 100 times more effective than TPA in increasing MCA-initiated transformation of BALB/3T3 cells, whereas dihydroteleocidin B and TPA were equally effective in inhibiting the binding of EGF and PDBu to cell receptors (*5*). However, TPA has strong activity in stimulating DNA synthesis in arrested cells, whereas debromoaplysiatoxin has no activity at all. This suggests that the cause of the inability of debromoaplysiatoxin to increase transformation is different from that of TPA. There may be qualitative differences in the binding of aplysiatoxin and debromoaplysiatoxin to receptors. The aplysiatoxin-debromoaplysiatoxin pair provides clues important for elucidating the factors (or steps) involved in tumor promotion and the functional role of the hydrophilic region of promoters.

KYOICHI SHIMOMURA
MARY G. MULLINIX
TAKEO KAKUNAGA*
Cell Genetics Section, Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205
HIROTA FUJIKI
TAKASHI SUGIMURA
National Cancer Center Research Institute, Tokyo, Japan

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* Address reprint requests to T.K.

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Nitrous Oxide Production in Nearshore Marine Sediments

Abstract. *Coastal marine sediments are shown to be a net source of nitrous oxide. The rates of nitrous oxide flux from sediments in Narragansett Bay, Rhode Island, ranged from 20 to more than 900 nanomoles per square meter per hour. Sediments from a eutrophic area had higher rates of net nitrous oxide production than sediments from relatively unpolluted sites. The benthic nitrous oxide source exceeds the nitrous oxide source to the bay from sewage treatment plant effluent.*

Recognition of the role of N_2O in the destruction of stratospheric O_3 (*1*) and in the radiative heat budget of the troposphere (*2*) has led to increased interest in the sources, sinks, and biogeochemical cycles of this trace gas. Anthropogenic increases in the global production rate of N_2O have been predicted as a consequence of increasing use of nitrogen fertilizer (*3*) and the combustion of fossil fuels (*4, 5*). The marine environment plays a major part in the global cycling of N_2O, with sources identified in surface ocean waters (*6–9*) and sinks in low-oxygen environments (*7–10*). Although it has been suggested that marine sediments may be net sinks or sources of N_2O (*8, 11*), as far as we know, no direct measurements of the N_2O flux to or from marine sediments have been made. We summarize here the results of measurements of N_2O fluxes from nearshore marine sediments taken from Narragansett Bay, Rhode Island, over an annual cycle, demonstrate that N_2O production per unit area is higher from polluted sediments than from relatively unpolluted sediments, assess the importance of benthic N_2O production relative to other terms in the nitrogen budget for the bay, and compare benthic N_2O production to estimated N_2O inputs to the bay from sewage treatment plant (STP) effluent.

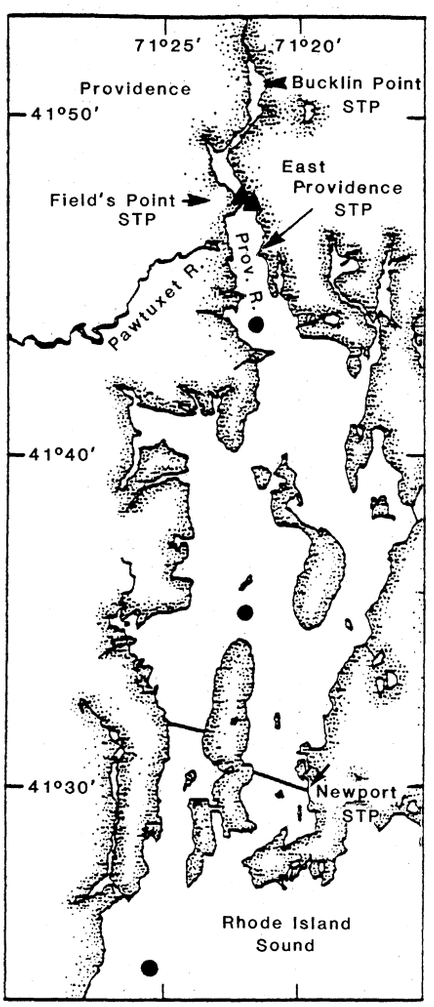

Fig. 1. Sediment (●) and water (▲) sampling stations in Narragansett Bay. The arrows indicate the points of discharge by four major sewage treatment plants (STP). Field's Point STP serves 200,000 people, Bucklin Point STP 95,700, East Providence STP 42,000, and Newport STP 39,000.

Sediment cores were collected between July 1978 and November 1979 by scuba divers from three stations in the Narragansett Bay area (Fig. 1). These stations lie along gradients in salinity, water depth, sediment organic content, silt-clay content, O_2 consumption, NH_4^+ production, pore-water H_2S concentration, and dominant macrofauna (Table 1). The cores were incubated in gastight chambers (*12*). The N_2O was measured in the water-equilibrated gas phase over the cores by electron-capture-detection gas chromatography (*12*), and N_2O production was calculated from the difference in the concentration of N_2O in sequential samples taken during the first 7 hours after the water was changed over a core. Sampling intervals were generally from 1 to 3 hours. Two to five flux measurements were made on each core.

There was a net flux of N_2O from the sediments in all cores from all three stations (Fig. 2), ranging from 19 nmole m^{-2} $hour^{-1}$ at the mid-Bay station to more than 900 nmole m^{-2} $hour^{-1}$ at the Providence River station, located close to numerous sources of sewage pollution. The N_2O production for these two stations showed an approximately exponential increase with increasing temperature. This temperature relation was more pronounced in the Providence River sediments (Q_{10} = 9.4) than in the mid-Bay sediments (Q_{10} = 2.3) (Q_{10} is the increase in the rate of chemical reaction for each 10°C increase in temperature). For the two experiments in which Rhode