Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1768–1771, March 1988
Biochemistry

Okadaic acid: An additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter

(tumor promotion/ornithine decarboxylase/two-stage mouse skin carcinogenesis)

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ABSTRACT **Okadaic acid is a polyether compound of a C_{38} fatty acid, isolated from a black sponge, *Halichondria okadai*. Previous studies showed that okadaic acid is a skin irritant and induces ornithine decarboxylase (OrnDCase; 3-hydroxyl-L-glutamate 1-carboxy-lyase, EC 4.1.1.17) in mouse skin 4 hr after its application to the skin. This induction was strongly inhibited by pretreatment of the skin with 13-*cis*-retinoic acid. A two-stage carcinogenesis experiment in mouse skin initiated by a single application of 100 μg of 7,12-dimethylbenz[*a*]anthracene (DMBA) and followed by application of 10 μg of okadaic acid twice a week revealed that okadaic acid is a potent additional tumor promoter: tumors developed in 93% of the mice treated with DMBA and okadaic acid by week 16. In contrast, tumors were found in only one mouse each in the groups treated with DMBA alone or okadaic acid alone. An average of 2.6 tumors per mouse was found in week 30 in the group treated with DMBA and okadaic acid. Unlike phorbol 12-tetradecanoate 13-acetate (TPA), teleocidin, and aplysiatoxin, okadaic acid did not inhibit the specific binding of [^{3}H]TPA to a mouse skin particulate fraction when added up to 100 μM or activate calcium-activated, phospholipid-dependent protein kinase (protein kinase C) *in vitro* when added up to 1.2 μM. Therefore, the actions of okadaic acid and phorbol ester may be mediated in different ways. These results show that okadaic acid is a non-TPA-type tumor promoter in mouse skin carcinogenesis.**

Non-TPA (phorbol 12-tetradecanoate 13-acetate)-type tumor promoters of mouse skin are defined as tumor promoters that do not bind to phorbol ester receptors in cell membranes (1, 2). Palytoxin and thapsigargin are non-TPA-type tumor promoters (3, 4). Since these compounds do not activate calcium-activated, phospholipid-dependent protein kinase (protein kinase C) *in vitro* (3, 4), non-TPA-type tumor promoters are thought to act through different pathways from TPA-type tumor promoters such as phorbol esters, teleocidin, and aplysiatoxin (1, 2). Moreover, palytoxin and thapsigargin may act through different pathways in cells, since palytoxin does not induce histidine decarboxylase (HisDCase; L-histidine carboxy-lyase, EC 4.1.1.22) in mouse skin (3) but thapsigargin does (4). These findings also indicate that the actions of these two non-TPA-type tumor promoters might involve different receptors. We wondered whether there might be other non-TPA-type tumor promoters with different actions from palytoxin and thapsigargin and so carried out screening for previously unreported non-TPA-type tumor promoters.

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Okadaic acid, a toxic polyether compound, was isolated from a black sponge, *Halichondria okadai*, and a sponge, *Halichondria melanodocia*, by Tachibana *et al.* (5). Okadaic acid and dinophysistoxin-1 (35-methylokadaic acid; Fig. 1) are implicated as causative agents of diarrhetic shellfish poisoning resulting from ingestion of mussels and scallops (6). Okadaic acid and dinophysistoxin-1 are thought to be synthesized in marine dinoflagellates and to accumulate in marine sponges and the digestive glands of mussels or scallops (7).

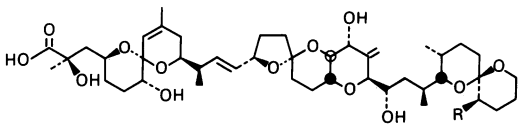
During screening for potential tumor promoters, we found that okadaic acid induces irritation of mouse ear and ornithine decarboxylase (OrnDCase; 3-hydroxyl-L-glutamate 1-carboxy-lyase, EC 4.1.1.17) in mouse skin, the inductions showing the same time courses as that by teleocidin (8). However, okadaic acid did not induce adhesion of human promyelocytic leukemia cells (HL-60 cells) or inhibit specific binding of [^{3}H]TPA to a mouse particulate fraction or activate protein kinase C *in vitro*. These findings were not consistent with the effects of TPA-type tumor promoters. A two-stage carcinogenesis experiment revealed that okadaic acid has potent tumor-promoting activity.

MATERIALS AND METHODS

Chemicals. Okadaic acid was isolated from a black sponge, *Halichondria okadai*, collected off the coast in Mie Prefecture, Japan. The material was obtained by methanolic extraction as described by Tachibana *et al.* (5) with some modifications. Briefly, okadaic acid was purified from the methanolic extract as follows: extraction with ethyl acetate, column chromatography twice on silicic acid (BW-820 MH, Fuji-Davison, Aichi, Japan), reverse-phase column chromatography on Develosil Lop ODS (Nomura, Aichi, Japan), and finally recrystallization from acetone. Teleocidin was isolated from *Streptomyces mediocidicus* (9). 7,12-Dimethylbenz(*a*)anthracene (DMBA) was purchased from Sigma. DL-[1-^{14}C]Ornithine monohydrochloride, [20-^{3}H(N)]TPA, and L-[*carboxyl*-^{14}C]histidine were obtained from New England Nuclear. [γ-^{32}P]ATP was obtained from Amersham.

Two-Stage Carcinogenesis Experiments. Initiation was carried out by application of 100 μg of DMBA in 0.1 ml of acetone to the skin of the back of 8-week-old female CD-1 mice (10). After 1 week, 10 μg of okadaic acid in 0.1 ml of acetone was applied to the skin twice a week until week 30. Two control groups were treated with DMBA alone and okadaic acid alone. In addition, a positive control group was treated with DMBA and 2.5 μg of teleocidin. Each group consisted of 15 mice, and the numbers of tumors of 1 mm or

Abbreviations: DMBA, 7,12-dimethylbenz(*a*)anthracene; HisDCase, histidine decarboxylase; OrnDCase, ornithine decarboxylase; TPA, phorbol 12-tetradecanoate 13-acetate.



	R
Okadaic acid	H
Dinophysistoxin-1	CH_3

FIG. 1. Structures of okadaic acid and dinophysistoxin-1.

more in diameter were counted weekly. All of the mice were sacrificed in week 30 of tumor promotion, and the tumors were examined histologically.

Inhibition of Specific [^{3}H]TPA Binding. Test compounds were incubated with 4 nM [^{3}H]TPA and 100 μg of a mouse particulate fraction for 2 hr at 0°C as described (11, 12). Nonspecific binding was measured in the presence of a 500-fold excess of unlabeled TPA. The specific binding of [^{3}H]TPA to 100 μg of particulate fraction was expressed as 100%.

Activation of Protein Kinase C. Protein kinase C was partially purified from mouse brain by DEAE-cellulose column chromatography (13). Enzyme activity was expressed as $^{32}P_i$ incorporation into histone H1 from [γ-^{32}P]ATP as described (14).

Induction of HL-60 Cell Adhesion. Cell adhesion was determined after incubating cells with a test compound for 48 hr. Details of the assay have been reported (15).

Irritant Test. Various amounts of okadaic acid were dissolved in 10 μl of acetone and applied to the inside of the ear of female CD-1 mice (Japanese Charles River, Kanagawa, Japan). The extent of irritation was expressed as the ID_{50}^{24} value as described (16, 17).

Induction of OrnDCase. Test compounds were dissolved in 0.2 ml of acetone and applied to the skin of the back of mice. OrnDCase activity was determined as reported (15, 18).

Induction of HisDCase. Compounds in 0.1 ml of acetone were applied to half the back (2 × 2 cm) of mice, and crude enzyme extracts were obtained from the skin 13 hr later, as described (4, 19). HisDCase activity was expressed as pmol of CO_2 released per mg of protein per 1 hr of incubation.

RESULTS

Tumor-Promoting Activity of Okadaic Acid in a Two-Stage Carcinogenesis Experiment. The effect of okadaic acid on tumor formation is illustrated in Fig. 2. The percentages of tumor-bearing mice in four experimental groups were recorded up to week 30. In the group treated with DMBA and okadaic acid, five tumors were found in 4 of 15 mice in week 5, and the tumor incidence was 93% in week 16. The percentage of tumor-bearing mice decreased slightly after week 17 and was 80.0% in week 30. In the group treated with DMBA and teleocidin, all mice had tumors in week 15. The average numbers of tumors per mouse in the groups treated with DMBA and okadaic acid and with DMBA and teleocidin were 2.6 and 6.8, respectively. In the groups treated with DMBA alone and okadaic acid alone, the incidences of tumors were each 6.7% (Fig. 2).

Table 1 shows histological findings on all 39 tumors in the group treated with DMBA plus okadaic acid and 93 of the 95 tumors in the group treated with DMBA plus teleocidin. Most of the tumors in both groups were papillomas. Only one tumor in the group treated with DMBA plus okadaic acid was identified as a sarcoma. The percentage incidences of papillomas and carcinomas were 92.3% and 5.1% in the group treated with DMBA plus okadaic acid and 95.7% and

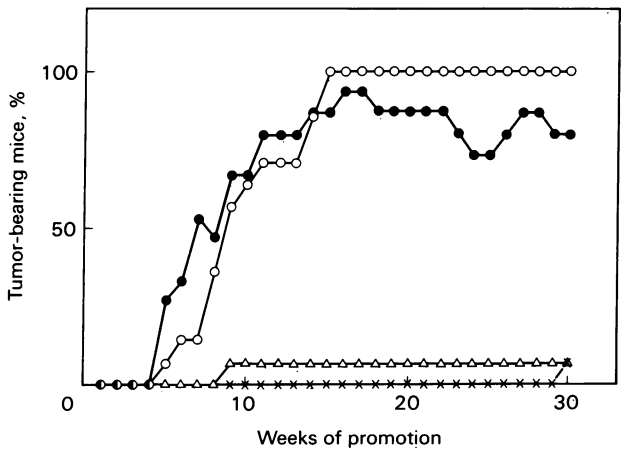


FIG. 2. Tumor-promoting activities of okadaic acid and teleocidin. Groups were treated with DMBA and okadaic acid (●), with DMBA and teleocidin (○), with DMBA alone (△), and with okadaic acid alone (×).

4.3% in the group treated with DMBA plus teleocidin. Thus, okadaic acid and teleocidin showed about the same potencies for production of skin papillomas and carcinomas during 30 weeks of tumor promotion. Since the molar ratio of the applied amounts of teleocidin to okadaic acid was 1:2, the tumor-promoting activity of okadaic acid seems to be nearly that of teleocidin. Okadaic acid is the most potent of the known non-TPA-type tumor promoters in mouse skin.

Comparison of the Biological and Biochemical Effects of Okadaic Acid and Other Non-TPA-Type Tumor Promoters. The specific binding of [^{3}H]TPA to a mouse particulate fraction was not inhibited by okadaic acid at concentrations of up to 100 μM, whereas teleocidin did inhibit this binding (2, 9) (Fig. 3). Protein kinase C was not activated by okadaic acid at concentrations up to 1.2 μM, whereas teleocidin induced phosphorylation of histone H1 by activation of protein kinase C (Fig. 4) (14). Furthermore, okadaic acid at concentrations up to 25 nM did not induce adhesion of HL-60 cells; at higher concentrations, it had cytotoxic effects on the cells. These three negative biological responses to okadaic acid were also observed with two other non-TPA-type tumor promoters (3, 4).

Okadaic acid is a skin irritant on mouse ear. The irritant activity of okadaic acid was almost the same as those of two other non-TPA-type tumor promoters. The ID_{50}^{24} of okadaic acid was 50 pmol per ear, whereas those of palytoxin and thapsigargin were 8 and 10 pmol per ear, respectively (3, 4, 8). These results indicate that okadaic acid is a potent skin irritant, like two other non-TPA-type tumor promoters and also TPA-type tumor promoters (1, 2). As we have reported (8), okadaic acid induces OrnDCase activity in mouse skin in a maximum time of 4 hr after its application, and this induction is strongly inhibited by pretreatment of the skin with 13-*cis*-retinoic acid. Fig. 5 shows the biphasic dose–response curve for induction of OrnDCase, determined 4 hr after application of okadaic acid. Maximum OrnDCase activity of 1.38 nmol of CO_2 per mg of protein per 30 min of incubation was induced by a single application of 10 μg (12.4

Table 1. Histological findings in skin tumors in mice initiated with DMBA

	No. of tumors (%)		
Tumor promoter	Papillomas	Squamous cell carcinomas	Sarcoma
Okadaic acid	36 (92.3)	2 (5.1)	1 (2.6)
Teleocidin	89 (95.7)	4 (4.3)	

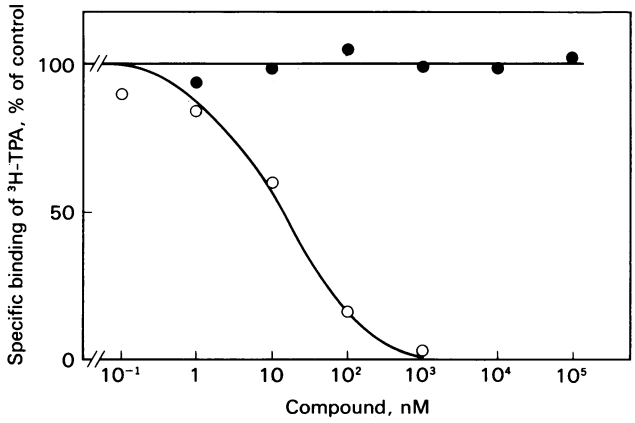


FIG. 3. Inhibition of specific [^{3}H]TPA (^{3}H-TPA) binding to a mouse particulate fraction. ●, Okadaic acid; ○, teleocidin.

nmol) of okadaic acid. Interestingly, although palytoxin and thapsigargin induced a strong inflammatory response, neither could induce OrnDCase in mouse skin (3, 4). Okadaic acid induced HisDCase in mouse skin. Application of 10 μg of okadaic acid induced the enzyme activity to a level of 143 pmol of CO_2 per mg of protein per 1 hr of incubation 13 hr after its application. The inductive activity of okadaic acid was approximately the same as that of thapsigargin (4). Palytoxin did not induce any HisDCase activity (3).

DISCUSSION

This paper reports the potent tumor promoting activity of okadaic acid in a two-stage mouse skin carcinogenesis experiment. Its effect was compared with those of two other non-TPA-type tumor promoters: okadaic acid was much less toxic to animals than palytoxin (3) and had much higher biochemical activity than thapsigargin (4). Application of 10 μg of okadaic acid to the skin of mice induced tumors in 93% of the mice in week 16. Thus, okadaic acid was a stronger non-TPA-type tumor promoter than palytoxin or thapsigargin. The induction of OrnDCase in mouse skin by okadaic acid might be involved in this high tumor-promoting activity. OrnDCase activity appears to be required for clonal tumor expansion in mouse epidermis (18). Table 2 shows a comparison of the biological and biochemical effects of the three non-TPA-type tumor promoters. These three non-TPA-type tumor promoters all had different effects. These differences suggest that these non-TPA-type tumor promoters act on the

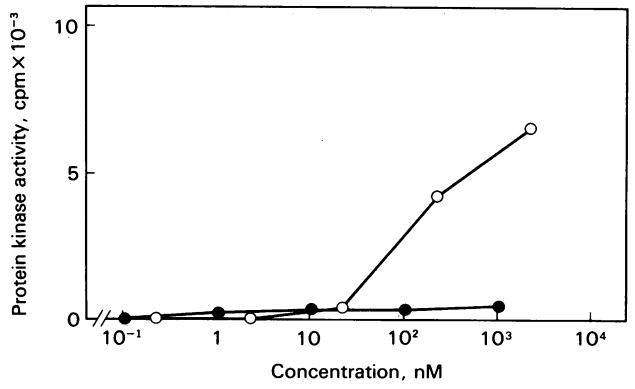


FIG. 4. Effects of okadaic acid and teleocidin on the activity of protein kinase C *in vitro*. ●, Okadaic acid; ○, teleocidin. Protein kinase C (5 μg) from mouse brain was incubated with test compounds, 5 μg of phosphatidylserine and 20 μM $CaCl_2$ in a total volume of 0.25 ml.

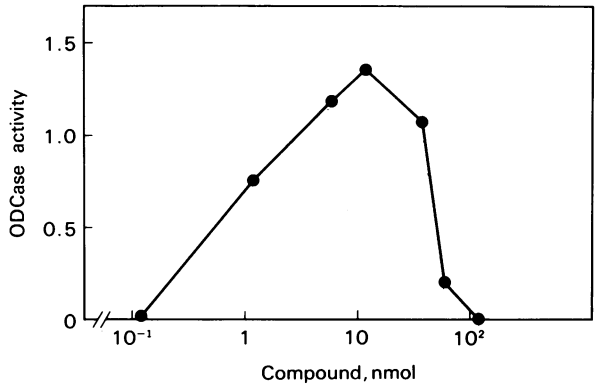


FIG. 5. Dose–response curve of OrnDCase (ODCase) induction by okadaic acid. OrnDCase activity is shown in nmol of CO_2 per mg of protein per 30 min of incubation.

cells through different pathways. Recently, we demonstrated the specific binding of [^{3}H]okadaic acid to a particulate fraction and a soluble fraction of mouse skin (H.F., unpublished results).

Dinophysistoxin-1 (35-methylokadaic acid), shown in Fig. 1, was isolated from mussels and scallops (6), and okadaic acid and dinophysistoxin-1 were isolated from a black sponge, *Halichondria okadai* (K.Y., M.O., and K.W., unpublished results). During the purification process, okadaic acid and dinophysistoxin-1 were finally separated by medium-pressure liquid chromatography on Develosil Lop ODS. Dinophysistoxin-1 shows as strong irritant activity on mouse ear and induction of OrnDCase in mouse skin as okadaic acid (unpublished results). Thus, dinophysistoxin-1 also may be a potent non-TPA-type tumor promoter in mouse skin.

Okadaic acid and dinophysistoxin-1 are products of marine dinoflagellates (7), and okadaic acid and its derivatives are widely distributed in marine organisms. There have been many cases of diarrhea due to eating mussels and scallops (7). Intubation of okadaic acid into the stomach of mice or rats caused diarrhea with accumulation of a large volume of fluid in the stomach, small intestine, and colon (20). Recently we demonstrated that okadaic acid induced OrnDCase in the glandular stomach of rats 4 hr after its intubation (unpublished results). Okadaic acid and dinophysistoxin-1 might act also as stomach tumor promoters.

Okadaic acid and dinophysistoxin-1 are threats to public health and to the shellfish industry in Japan, Hawaii, Chile, Norway, Holland, and Spain. Methods have been developed for determining the contents of these toxins in marine organisms (7). Hokama *et al.* (21) reported the development of a Poke Stick Test, which is based on an enzyme immunoassay procedure. This method can be used to detect nanogram levels of okadaic acid. Lee *et al.* (22) measured the fluorescence intensity of the toxins after their reaction with 9-anthryldiazomethane, a method by which it was possible to detect 1–80 ng of toxins. These methods will be useful in determining the contents of these toxins in daily foods from marine organisms.

The mechanisms of the early actions of TPA-type tumor promoters TPA, teleocidin, and aplysiatoxin are known to

Table 2. Differential pattern of effects induced by three non-TPA-type tumor promoters

Tumor promoter	Irritation	OrnDCase	HisDCase	Refs.
Okadaic acid	+	+	+	
Palytoxin	+	−	−	1, 3
Thapsigargin	+	−	+	2, 4

involve binding to phorbol ester receptors and activation of protein kinase C (14, 23), but the mechanisms of action of the non-TPA-type tumor promoters palytoxin and thapsigargin are unknown. Recently, we found that okadaic acid induces hyperphosphorylation of a 60-kDa nucleolar protein in simian virus 80 cells (a simian virus 40-transformed Fanconi cell line), whereas okadaic acid tetramethyl ether does not induce any phosphorylation (24). Okadaic acid tetramethyl ether, a synthetic derivative of okadaic acid, does not induce irritation of mouse ear or OrnDCase in mouse skin (8). The 60-kDa protein is a fragment of the phosphoprotein C23 (nucleolin) (24). Studies on the mechanism of action of okadaic acid as an additional non-TPA-type tumor promoter should be interesting.

This work was supported in part by Grants-in-Aid for Cancer Research and Scientific Research from the Ministry of Education, Science and Culture; a grant from the fund for a Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan; and grants from the Foundation for Promotion of Cancer Research and the Princess Takamatsu Cancer Research Fund.

1. Fujiki, H., Suganuma, M., Tahira, T., Yoshioka, A., Nakayasu, M., Endo, Y., Shudo, K., Takayama, S., Moore, R. E. & Sugimura, T. (1984) in *Cellular Interactions by Environmental Tumor Promoters*, eds. Fujiki, H., Hecker, E., Moore, R. E., Sugimura, T. & Weinstein, I. B. (Japan Sci. Soc., Tokyo; VNU Science, Utrecht, The Netherlands), pp. 37–45.
2. Fujiki, H. & Sugimura, T. (1987) *Adv. Cancer Res.* **49,** 223–264.
3. Fujiki, H., Suganuma, M., Nakayasu, M., Hakii, H., Horiuchi, T., Takayama, S. & Sugimura, T. (1986) *Carcinogenesis* **7,** 707–710.
4. Hakii, H., Fujiki, H., Suganuma, M., Nakayasu, M., Tahira, T., Sugimura, T., Scheuer, P. J. & Christensen, S. B. (1986) *J. Cancer Res. Clin. Oncol.* **111,** 177–181.
5. Tachibana, K., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y. & Schmitz, F. J. (1981) *J. Am. Chem. Soc.* **103,** 2469–2471.
6. Murata, M., Shimatani, M., Sugitani, H., Oshima, Y. & Yasumoto, T. (1982) *Bull. Jpn. Soc. Sci. Fish.* **48,** 549–552.
7. Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G. K. & Clardy, J. (1985) *Tetrahedron* **41,** 1019–1025.
8. Fujiki, H., Suganuma, M., Suguri, H., Yoshizawa, S., Ojika, M., Wakamatsu, K., Yamada, K. & Sugimura, T. (1987) *Proc. Jpn. Acad. Ser. B* **63,** 51–53.
9. Fujiki, H. & Sugimura, T. (1983) *Cancer Surv.* **2,** 539–556.
10. Fujiki, H., Suganuma, M., Matsukura, N., Sugimura, T. & Takayama, S. (1982) *Carcinogenesis* **3,** 895–898.
11. Ashendel, C. L. & Boutwell, R. K. (1981) *Biochem. Biophys. Res. Commun.* **99,** 543–549.
12. Suganuma, M., Fujiki, H., Tahira, T., Cheuk, C., Moore, R. E. & Sugimura, T. (1984) *Carcinogenesis* **5,** 315–318.
13. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 13341–13348.
14. Fujiki, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y. & Sugimura, T. (1984) *Biochem. Biophys. Res. Commun.* **120,** 339–343.
15. Fujiki, H., Mori, M., Nakayasu, M., Terada, M., Sugimura, T. & Moore, R. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3872–3876.
16. Hecker, E. (1971) *Methods Cancer Res.* **6,** 439–484.
17. Fujiki, H., Mori, M., Nakayasu, M., Terada, M. & Sugimura, T. (1979) *Biochem. Biophys. Res. Commun.* **90,** 976–983.
18. O'Brien, T. G., Simsiman, R. C. & Boutwell, R. K. (1975) *Cancer Res.* **35,** 1662–1670.
19. Watanabe, T., Taguchi, Y., Kitamura, Y., Tsuyama, K., Fujiki, H., Tanooka, H. & Sugimura, T. (1982) *Biochem. Biophys. Res. Commun.* **109,** 478–485.
20. Terao, K., Ito, E., Yanagi, T. & Yasumoto, T. (1986) *Toxicon* **24,** 1141–1151.
21. Hokama, Y., Ougi, A. M., Honda, S. A. A. & Matsuo, M. K. (1985) *5th Int. Coral Reef Congr.* **4,** 449–455.
22. Lee, J. S., Yanagi, T., Kenma, R. & Yasumoto, T. (1987) *Agric. Biol. Chem.* **51,** 877–881.
23. Nishizuka, Y. (1984) *Nature (London)* **308,** 693–698.
24. Martin, Th., Fujiki, H. & Issinger, O.-G. (1987) *Biol. Chem. Hoppe-Seyler*, **368,** 1079.