BIOLOGICAL ACTIVITIES OF NOVEL POLYETHER ANTIFUNGALS, GAMBIERIC ACIDS A AND B FROM A MARINE DINOFLAGELLATE *Gambierdiscus toxicus*

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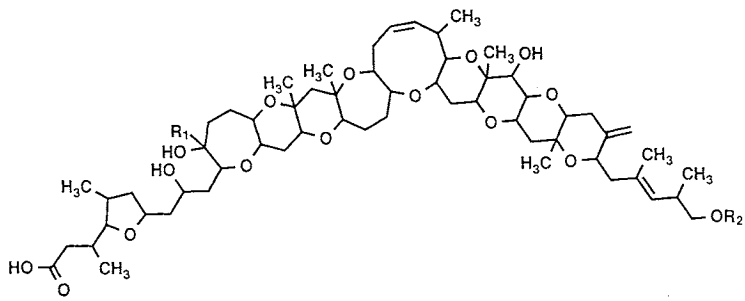
Over the past several years, many chemically and biochemically important polyethers[1] such as brevetoxin[2,3], ciguatoxin[4], maitotoxin[5] and okadaic acid[6] have been isolated from various marine microorganisms, especially from dinoflagellates. Many of those tested had antifungal activities[7]. During the course of further studies on new antifungal substances from phytoplankton, we isolated new polyethers with potent antifungal activity; gambieric acids A, B, C and D (Fig. 1) from a marine dinoflagellate *Gambierdiscus toxicus*[8,9]. In this paper, we report the antifungal activity of gambieric acids A and B together with some other biological properties.

Gambieric acids were purified from the filtered culture broth of *Gambierdiscus toxicus* as previously described[9,10]. The filtrate (1,000 liters) was passed through a column of polystyrene resin (Amberlite XAD-2). After elution with methanol, the concentrated active fraction was further purified by solvent partition and column chromatography and finally 2.5 mg of gambieric acid mixture was obtained. The yields of gambieric acids A, B and a mixture of C and D were 1.2×10^{-8}, 0.3×10^{-8} and 11.6×10^{-8}% by weight of the culture medium, respectively.

Gambieric acids were dissolved in methanol and the reference drug, amphotericin B (Bristol-Myers Squibb Co., Ltd., Tokyo, Japan) was dissolved in dimethyl sulfoxide. Minimum inhibitory concentration (MIC) was defined as the lowest drug concentration resulting in complete inhibition of visible growth. Sub-MIC was also defined as the lowest drug concentration of each drug showing incomplete (partial) inhibition. MIC and sub-MIC determinations of both drugs were performed by a previously described method[9]. Micro-broth dilution microplates (Sumitomo Chemical Co., Osaka, Japan) were prepared to which two-fold serial dilutions of gambieric acids A and B or amphotericin B were added. Yeast nitrogen base (Difco) plus 1%

Fig. 1. Structures of gambieric acids A, B, C and D.



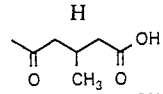
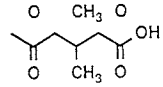
	R_1	R_2
Gambieric acid A	H	H
Gambieric acid B	CH_3	H
Gambieric acid C	H	
Gambieric acid D	CH_3	

Table 1. Antifungal activity of gambieric acids A (GA-A) and B (GA-B) in comparison with that of amphotericin B (AMPH).

Microorganism	MIC and sub-MIC value (μg/ml)		
	GA-A	GA-B	AMPH
Aspergillus fumigatus No. 184	0.39 (0.025)[a]	0.78 (0.05)	3.13
A. niger IFM 40606	0.20 (0.025)	0.2 (0.025)	3.13
A. oryzae IFM 40607	3.13 (<0.01)	6.25 (0.025)	6.25
Epidermophyton floccosum IFM 40770	3.13 (<0.01)	1.56 (<0.01)	3.13
Paecilomyces variotii IFM 30539	0.78 (0.01)	0.78 (0.05)	3.13
Penicillium chrysogenum Q176	1.56 (0.1)	1.56 (0.05)	6.25
P. citrinum IAM 7003	3.13 (<0.01)	3.13 (<0.01)	≧12.5
Trichophyton mentagrophytes IFM 45110	0.78 (<0.01)	0.78 (<0.01)	1.56

MIC was determined by broth micro-dilution method using buffered yeast nitrogen base medium with 1% glucose.

[a] Numbers in parentheses mean sub-MIC values.

glucose whose medium pH was adjusted to 7.0 by 1/20 M MOPS buffer was used for fungi. A sensitivity test broth (Eiken, Tokyo, Japan) was used for bacteria. MIC and sub-MIC were determined after 48 hours incubation at 37°C for bacteria and yeast type fungi, and 96 hours at 27°C for filamentous fungi. *In vitro* cytotoxic activity was determined using a cultured cell line of P388 mouse lymphoma.

Gambieric acids were not active against the bacteria tested, which included *Bacillus subtilis* PCI 219, *Staphylococcus aureus* 209P, *Micrococcus luteus* IFM 2066 and *Escherichia coli* NIHJ-JC2 at the concentration of 12.5 μg/ml, which was the maximum concentration used in these studies. Gambieric acids A and B were also not active against the yeast type fungi *Candida albicans* IFM 1001, *Cryptococcus neoformans* CUH-12 and *Saccharomyces cerevisiae* IFM 40022, although they showed potent activities against filamentous fungi. The MIC values of tested fungi are shown in Table 1 in comparison with those of amphotericin B. *Aspergillus niger* and *Aspergillus fumigatus* were the most sensitive species and both were inhibited at concentrations of 0.2 to 0.78 μg/ml, respectively. Interestingly, gambieric acids showed incomplete inhibition leading to sub-MIC values, although amphotericin B showed only complete inhibition in the present assay system. When the sub-MIC values of the drugs were compared with the MIC values of amphotericin B, the difference was much more prominent, and a more than 60 to 1,000 times difference in the activity was confirmed between gambieric acids and amphotericin B. Among the tested filamentous fungi, *Epidermophyton floccosum*, *Trichophyton mentagrophytes* and *Penicillium citrinum* showed lower sub-MIC values, and as low as 0.01 μg/ml sub-MIC was observed. To further confirm the potent antifungal activity of gambieric acid, the activity was also determined by paper disc method. Namely, an appropriate amount of gambieric acid was applied to a filter paper disc 8 mm in diameter. The paper disc was placed on agar media (Yeast nitrogen base medium plus 1% glucose) seeded with the spores of *Aspergillus niger*. After incubation at 37°C for 48 hours, inhibiting circles were observed around the disc. Gambieric acids showed an inhibition zone as low as 10 ng/disc, but such antifungal activity was not observed less than 20 μg/disc of amphotericin B. Thus, it was found that the antifungal activity of gambieric acids is 50 to 2,000 times higher than that of amphotericin B in the present experimental condition.

The cytotoxicity test of gambieric acids A and B were accomplished as follows: Mouse lymphoma cells (P388) were grown in a tissue culture medium of RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml of streptomycin. A 100 μl of portion of the culture containing approximately 5,000 cells was added to each well of a flat-bottomed microplate (Falcon 3072). Subsequently, aliquots of serially diluted solutions of gambieric acids in methanol were added to the wells. Care were taken to keep methanol concentration in the wells below 0.5%. After incubation with gambieric acids for 68 hours at 37°C in the atmosphere of 5% CO_2, 1 μCi of ^{3}H-thymidine, ^{14}C-leucine or ^{3}H-uridine (New England Nuclear) dissolved in 20 μl of the culture medium was added to each well. The each cell was incubated for another 4 hours and then transferred onto filter paper by using a cell harvester. The incorporation ratio of each isotope was determined

with a liquid scintillation counter. When the cytotoxicity of gambieric acid A against P388 cultured cells was determined by the incorporation of labeled compounds into P388 cells, IC_{50} values were 490; 480 and 120 ng/ml, respectively. Cytotoxic activity of gambieric acid B was slightly higher than that of gambieric acid A and the IC_{50} values were 370, 420 and 70 ng/ml, respectively. Since these inhibitory effects of gambieric acids against macromolecular syntheses occur at almost the same dose level, they may be a secondary effect. Although the *in vivo* toxicity of gambieric acids could not be tested at high doses due to the difficulty in obtaining enough sample, the dose of 1 mg/kg (intraperitoneally administered) for both gambieric acids A and B did not show either toxicity or abnormal reaction in *ddY* mice.

We had reported the antifungal activity of various polyethers from dinoflagellate origins[7]. Our present studies with gambieric acids adds new experimental data showing that most polyethers from dinoflagellate origins have potent antifungal activities. New polyethers, goniodomin A[11] and amphidinol[12] from dinoflagellate *Goniodoma pseudogoniaulax* and *Amphidinium klebsii*, respectively, have also been reported. Therefore, dinoflagellates are expected to be a candidate for new sources of novel antifungal substances such as actinomycetes.

The higher antifungal activity of gambieric acids warrants further *in vivo* studies against fungal infections such as aspergillosis, since their toxicity to mice and cultured cells is moderate compared to other polyethers of dinoflagellate origin[2~5]. During these studies we also found that the antifungal activity of gambieric acids A and B was potentiated by ferric compounds such as $FeCl_3$ or $Fe_2(SO_4)_3$, although this potentiation was not exhibited by $CaCl_2$, KCl, NaCl, $MgCl_2$ or $ZnCl_2$ (data not shown). Since this potentiation was not prominent with ferrous compounds like $FeCl_2$ and $FeSO_4$, ferric ion may play an important role in the exhibition of the potentiation. Detailed studies on the mechanisms of the potentiation, coupled with the studies on mode of action have just begun in the second author's laboratory.

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