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Flavonoids and Stilbenes as Repellents against the Blue Mussel, *Mytilus edulis galloprovincialis*

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Abstract – We studied the attachment-repelling activity of flavonoids, stilbenes, and their glycosides against the blue mussel *Mytilus edulis galloprovincialis*. Molecular mechanics calculations have shown that the most stable conformations of potent repellents **29** and **32** were the same. On the basis of these results, structure-activity relationships of these compounds are discussed.

Keywords – flavonoids, stilbenes, blue mussel, *Mytilus edulis galloprovincialis*, attachment-repellents.

Introduction

Marine fouling caused by the attachment of marine sessile organisms such as barnacles and blue mussels to the surface of ships and to the pipes in a power plant has been creating many problems (Richmond and Seed, 1991). Organotin compounds and cuprous oxide have been used as effective antifouling agents to solve this problem. However, pollution caused by liquation of tin and copper from these antifouling compounds, and subsequent bioconcentration of these metals in marine organisms is of serious concern all over the world. Therefore the use of organometals is highly undesirable and there is a need to the search for non-toxic antifouling agents.

Flavonoids are a group of natural products mainly responsible for the coloring found in vascular plants. Flavonoids exhibit a wide range of biological activities and are potential anti-cancer, insect anti-feedants, and natural insecticides. We isolated kaempferol 3-*O*-(2,6-di-*O*-(*E*)-*p*-coumaryl-β-D-glucopy- ranoside), a new acylated kaempferol glucoside from *Quercus dentata* Thunberg (Yamashita *et al.*, 1989 a) and two novel monoterpene flavonol glycosides, resinosides, A and B from the leaves of *Eucalyptus resinifera* (Hyodo *et al.*, 1992). We also isolated stilbene glycosides, rhaphonticin 6-*O*-(*E*)-*p*-coumarate and polydatin 6-*O*-(*E*)-*p*-coumarate from the leaves of *Eucalyptus rubida* (Yamashita *et al.*,1989 b, and Etoh *et al.*, 1990). This suggested that a study of the repellent activity of flavonoids and stilbenes may be a fruitful exercise. In this paper we report on the repellent activity of a number of flavonoids, stilbenes, and their glycosides against the blue mussel *Mytilus edulis galloprovincialis*, and the structure-activity rel-

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ationship among these compounds.

Experimental

Tested compounds – All the flavonoids except **12, 14**, and **15** were purchased from Tokyo Kasei Co., Aldrich Co. and Wako Co. and then purified. Stilbene glycosides were synthesized according to the literature procedure (Etoh *et al.*, 1991). Sideroxylin (**12**) was isolated from *E. amplifolia* and identified based on spectral data (Wollenweber and Kohorst, 1981). Pinocembrin (**14**) and dimethylpinocembrin (**15**) were isolated from *E. signata* and identified by comparison with authentic samples (Bick *et al.*, 1972).

Test organisms – The blue mussels, *Mytilus edulis galloprovincialis* (Japanese name "Murasaki igai" 15-25 mm in shell length), were collected from the pylons wharfs at the Mochimune fishing harbour in Shizuoka city and maintained in an aquarium with running sea-water for at least a week before the experiments.

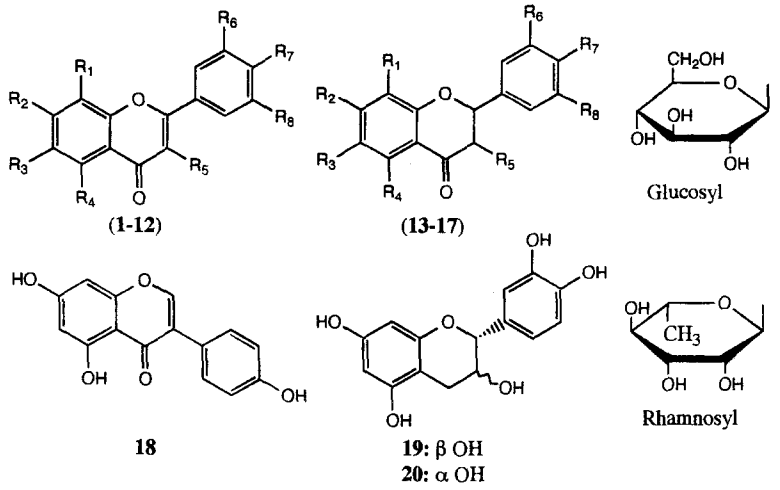
Attachment-repelling Activity – The attachment-repelling activity of the compounds was evaluated by an improved bioassay method (Ina *et al.*, 1989). The assay plate was prepared from a commercially available thick plate (Webron, 30×50×0.5 cm; Tokushu Seishi Co.) which was washed with methanol and dried before use. The sample zones (4 cm in diameter) were drawn a few centimeters apart, coated with a test sample dissolved in a solvent and then dried. Four test mussels were fixed around the edge of the circle, using a spacer made from a rubber stopper, with a cyano-acrylate adhesive. The assay plate was placed at the bottom of an aquarium provided with running seawater and left in the dark for 3 h. The mussels fixed themselves to the plate by attaching byssuses outside the sample zone. The unit of measurement was calculated by the following equation:

$$\text{unit} = \frac{\text{minimum dose of } CuSO_4 \text{ for } ++ \ (\mu\text{mmol/cm}^2)}{\text{minimum dose of sample for } ++ \ (\mu\text{mmol/cm}^2)}$$

Molecular mechanics calculations – Conformation analysis of **29** and **32** were performed using the Biosym Insight and Discover modeling software on an Indigo 2 work station of Silicon Graphics, Inc. The starting structures were generated by Insight II (Insight II User Guide, ver. 95.0, BIOSYM/ Molecular Simulations: San Diego, 1995) and the dihedral angles of the single bonds (marked with thick lines, Fig. 3) were rotated by an increment of 30° using Search-Compare procedure (Search-Compare User Guide, ver. 95.0, BIOSYM/Molecular Simulations: San Diego, 1995) affording 16273 and 19764 chemically plausible conformers of **29** and **32**, respectively. Out of these, 400 low energy conformers of each compound were selected. The structure optimization was carried using the CFF91 force field of the Discover program (Discover User Guide, ver. 95.0, BIOSYM/ Molecular Simulations: San Diego, 1995). The structures converged to the same conformation were selected to obtain 46 and 39 independent conformers of **29** and **32**, respectively. The conformation energies of global minimum structures of **29** and **32** were -30.119 kcal and -52.071 kcal/mole, respectively.

Results and discussion

Fourteen flavonoid aglycones, one isoflavonoid, and five flavonoid glycosides were tested for attachment-repelling activity against blue mussel, *Mytilus edulis galloprovincialis*. The repellent activities of flavonoids are summarized in Fig. 1. Among all the flavonoid aglycones tested, compound **11** was the most potent and showed repellent activity at unit 25 (25% of the activity of $CuSO_4$, a standard anti-foulant). An obvious correlation was observed between the attachment-repelling activity and the number and position of hy-



Comp.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Unit
1:	H	OH	H	OH	OH	H	OH	H	8
2:	H	OH	H	OH	OH	OH	OH	H	12
3:	H	OH	H	OH	OH	OH	OH	OH	8
4:	H	OH	H	OH	O-Rha	H	OH	H	10
5:	H	OH	H	OH	O-Glc	H	OH	H	5
6:	H	OH	H	OH	O-Rha	H	OH	OH	20
7:	H	OH	H	OH	O-Glc-Rha	H	OH	OH	19
8:	H	H	H	H	H	H	H	H	<4
9:	H	OH	H	H	H	H	H	H	<4
10:	H	H	H	H	OH	H	H	H	<4
11:	OH	OH	H	H	H	H	H	H	25
12:	CH_3	OCH_3	CH_3	OH	H	H	OH	H	--*
13:	H	OH	H	OH	H	H	OH	H	11
14:	H	OH	H	OH	H	H	H	H	20
15:	H	OCH_3	H	OCH_3	H	H	H	H	--**
16:	H	O-Glc-Rha	H	OH	H	H	OH	H	24
17:	H	H	H	H	H	H	H	H	<4
18:									9
19:									<4
20:									<4

*Inactive at unit 4, **Inactive at unit 10

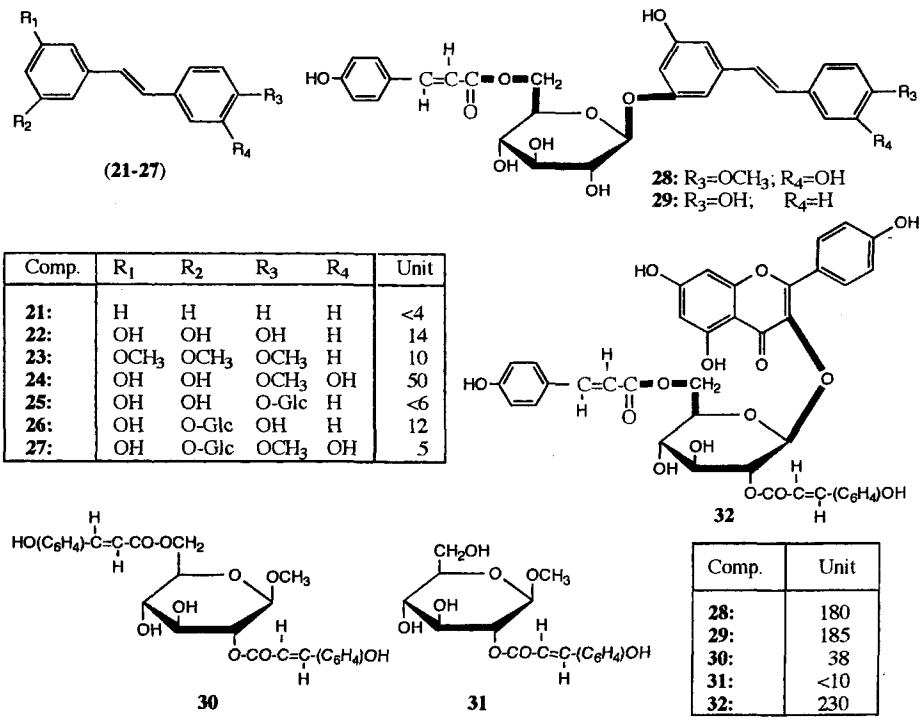
Fig. 1. Structures and Activity of Flavonoids.

droxyl groups. Generally, the presence of hydroxyls at positions 5 and 7 of the ring A and carbonyl group at position 4 of ring C was found to be essential requirement for these compounds to exhibit attachment-repelling activity. Compounds such as **8** and **16** which had no hydroxyl at all were the least active showing activity at unit 4 (less than 4% of the activity of $CuSO_4$). Pinocembrin (**14**) showed activity at unit 20 which was reduced when both the hydroxyls of ring A were replaced by methoxy groups as in **15**. Similarly, catechin (**19**) and epicatechin (**20**), which lacked the carbonyl group at C-4 also showed very weak activity at less than 4 units. Results from five flavonoid glycosides showed that glycosides which had rhamnosyl moiety were more active than those having glucosyl moiety. Kaempferol 3-*O*-rhamnoside (**4**) was almost two times more active than kaempferol 3-*O*-glucoside (**5**) showing the importance of rhamnosyl moiety. Similarly, Quercetrin (**6**) which had only rhamnosyl was as active as **7** which

had both the rhamnosyl and glucosyl moieties. Presence of two hydroxyls in ring B, in addition to hydroxyls of ring A seemed to enhance the activity as observed for **6** and **7** in comparison to **4** and **5**. Naringin (**16**) was more active than naringenin (**13**) showing the importance of glycosidic linkage. In fact, naringin was the most active repellent among the flavonoid glycosides (unit 24).

Stilbene (**21**) showed only weak attachment-repelling activity at a dose of 12.4 μmol /cm^2 (unit<4). Introduction of hydroxyl groups lead to enhanced repelling activity as was observed for 3,5,4'-trihydroxy stilbene (**22**) which showed activity at unit 14. Activity was diminished when all the hydroxyl groups were replaced with methoxy groups in **23**. However, introduction of an additional methoxy group significantly increased the repelling activity of rhapontigenin (**24**, unit 50). Introduction of either glycosidic linkage or coumaryl moiety alone did not appreciably effect the repelling activity. However, the activity was dramatically increased by introduction of glycosidic linkage and coumaryl moiety together as in **28, 29**, and **32**. Acylated kaempferol glucoside (**32**) was almost forty times more active than kaempferol glucoside (**5**) indicating the importance of coumaryl moiety. A comparison of activity of **30** and **31** also showed that **30** with two coumaryl groups was almost three times more active than **31** with only one coumaryl group.

It follows from the foregoing that the presence of multiple hydroxyl groups was necessary for these compounds to exhibit attachment-repelling activity. Presence of hydroxyl at positions 5 and 7 of ring A and carbonyl at position 4 of ring C of flavonoids was essential for activity. Flavonoids with two hydroxyl groups in each of rings, A and B were more active than others. We have earlier correlated antifouling activity with antimicrobial activity against selected microorganisms and attachment repelling activity against the blue mussel *Mytilus edulis galloprovin-*



Comp.	R_1	R_2	R_3	R_4	Unit
21:	H	H	H	H	<4
22:	OH	OH	OH	H	14
23:	OCH_3	OCH_3	OCH_3	H	10
24:	OH	OH	OCH_3	OH	50
25:	OH	OH	O-Glc	H	<6
26:	OH	O-Glc	OH	H	12
27:	OH	O-Glc	OCH_3	OH	5

Comp.	Unit
28:	180
29:	185
30:	38
31:	<10
32:	230

Fig. 2. Structures and Activity of Stilbenes and Coumarates.

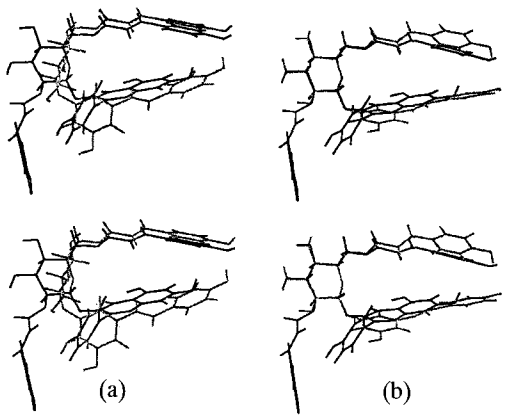


Fig. 3. (a) Two lowest energy Conformers of **29** (gray line) and **32** (thick black line); (b) Two most closely resembling Conformers of **29** and **32**.

cialis. The antimicrobial activity of alkyl phenols is thought to be brought about by their membrane perturbation action. We found that both the antimicrobial activity and membrane perturbation potency varied parabolically with increasing hydrophobicity of the *para* substituent. This parabolic change was attributed to a 'cut-off' phenomenon in the partitioning of phenols. These results indicated that the antifouling action of phenolic compounds might be related to their membrane perturbation potency (Etoh *et al.*, 1994a).

We have shown earlier that **28** was more active than any of its components taken individually or as a mixture (Etoh *et al.* 1991). Thus, stilbene (**24**) showed activity at unit 50, *p*-coumaric acid at unit 7 and glucose at unit<0.01. Their mixture (1:1:1) showed activity at unit<67. Similarly, **32** was also more active than its components, kaempferol (unit 8), and *p*-coumaric acid (Yamashita *et al.*, 1989 a). A mixture of these (1:2) was even less active. In order to understand more about the reasons for high activity of these compounds, we performed molecular mechanics calculations on **29** and **32** which have shown that the general view of the most stable conformation of these compounds resemble each other. As shown in Fig 3a, the spatial arrangement of the aglycon and the coumaryl substituent at position 6 are similar for the lowest energy conformers of both the compounds. However, the average plane of sugar moieties are approximately perpendicular to each other for two compounds. A combination of more closely related conformers was searched in the data set of finally obtained structures, and as shown in Fig. 3b, the two sugar rings superimpose each other and the two substituents also overlap well. These results show that full structure as well as conformation of natural products isolated earlier from *Eucalyptus and Quercus dentata* was necessary for these compounds to show repelling activity. As enzymes are associated with the adhesion of blue mussel (Etoh *et al.*, 1994b), it may be assumed on the basis of the foregoing, that they play a role in determining the attachment repelling activity also.

It can be concluded from the above discussion that although the presence of hydroxyls, carbonyl and rhamnoside is necessary, but the conformation as well as full structure of **29** and **32** is very important for high level of repellent activity of these compounds.

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