A Sulfonoglycolipid with Na^+, K^+-ATPase Inhibitory Activity, Produced by a Cultured Unique Diatom Symbiot Isolated from a Larger Foraminifera

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Abstract: **A unique diatom (*Entomoneis* sp.) was isolated from a larger foraminifera (*Marginopora vertebralis*) and was successfully mass cultured. 6-Sulfo-*O*-α-D-quinovopyranosyldiacylglycerol was isolated from this diatom as an Na^+,K^+-ATPase inhibitor.**

Marine microorganisms such as blue-green algae and dinoflagellates have been recognized as a valuable new source of pharmacologically and clinically useful compounds (1, 2). In this study, we have successfully isolated and mass cultured a new type of diatom (*Entomoneis* sp.), symbiotically associated within the body of a larger foraminifera (*Marginopora vertebralis*), and purified 6-sulfo-*O*-α-D-quinovopyranosyl-diacylglycerol with activity for Na^+,K^+-ATPase inhibition.

The diatom was isolated from the body of the *Marginopora vertebralis*, and successfully mass cultured for the first time in our laboratory. This diatom was considered to belong to the genus *Entomoneis* sp. from observation of the scanning electron micrograph (Fig. **1**). The taxonomic characteristics of this diatom were very similar to those of *Amphiprora paludosa*. *Entomoneis* sp. has been considered to be a subgroup of *Amphiprora*. But it is different from *Amphiprora paludosa* since this diatom has 50–60 striae in 10 μm and no "junction line" characteristic on the surface of the body.

The *n*-BuOH solubles of the MeOH extracts of the harvested cells of this diatom were repeatedly chromatographed to afford 6-sulfo-*O*-α-D-quinovopyranosyldiacylglycerol (**1**). The composition of the fatty acids was shown to be palmitate and myristate by mass spectroscopic analysis of the products from alkaline hydrolysis of **1**. The sugar part (**2**) was identical in all respects with 6-sulfo-*O*-α-D-quinovopyranosyl(1→1′)-glycerol (3, 4). The stereochemistry at C-2′ of the glycerol was established as *S*-configuration by comparison of the optical rotation, the chemical shifts, and the splitting patterns of the NMR signals to the data of Gustafson et al. (5). Sulfonoglycolipids were first described in the chemical literature by Benson et al. (6, 7) and members of this structural class were commonly referred to as sulfonoquinovosyl diacylglycerols. These sulfonoglycolipids are structural components of chloroplast membranes and occur widely in higher plants, algae,

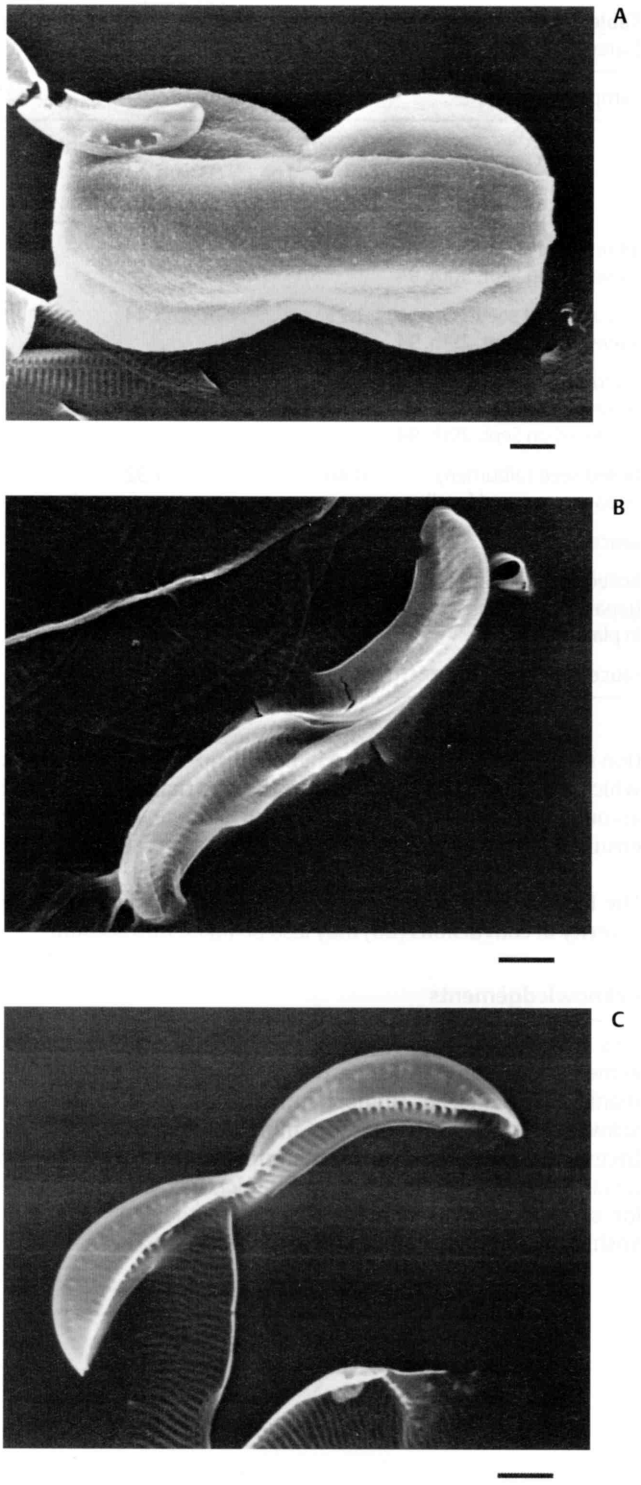
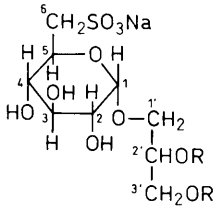


Fig. 1 The scanning electron micrograph of diatom symbiot isolated from *Marginopora vertebralis*. **A**: Girdle view of a whole frustule showing valves with deep constriction and cingulum. **B**: Valve view showing sigmoid canal raphe. **C**: Oblique view of the valve showing striae, basal fibulae and raphe fibulae. Scale bars = 1 μm.

and photosynthetic microorganisms (8, 9). Furthermore, it has been reported that sulfonoglycolipids were active against HIV-1 in cultured human lympoblastoid (5).



1 R = palmitoyl, myristoyl

2 R = H

Na^+,K^+-ATPase inhibitors such as ouabain have been studied extensively because of their physiologically important roles, such as the cardiotonic effect (10). In our experiments, **1** inhibited Na^+,K^+-ATPase activity in a concentration-dependent manner (Fig. **2**) with the 50% inhibitory concentration (IC_{50}) of 2×10^{-4} M without affecting Ca^{2+}-ATPase from skeletal muscle SR. In addition, **1** (10^{-5}–10^{-4} M) did not affect P388 cell culture and physiological functions of platelets.

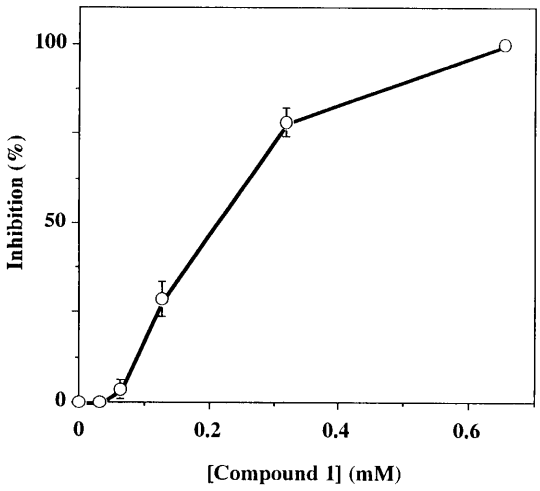


Fig. 2 Inhibitory effects of compound **1** on activities of brain Na^+,K^+-ATPase. The enzyme reaction was carried out at 37 °C for 10 min. Results are expressed as the mean ± S.E. of three experiments.

This symbiotic diatom has become an important medicinal natural resource because of its successful mass culture. Now further detailed pharmacological studies of **1** and determination of the species of this diatom are under investigation.

Materials and Methods

Cultivation, isolation, and identification: The larger foraminifera *Marginopola verteblaris* was crushed after being washed three times with sterilized sea water and once with 70% EtOH. A diatom symbiot was isolated by a capillary glass tube under the microscope.

Uni-algal culture of *Entomoneis* sp. was grown in 3-l glass bottles containing 2 l of sterilized sea water medium enriched with modified ESM supplement (11). After 10–14 days the cultured cells were harvested with glass filters (GF/F), Whatmann) to yield the cells.

The harvested cells from 500 l of culture were extracted with MeOH (500 ml × 3) to give a MeOH extract (25 g) which was partitioned with EtOAc/H_2O. Water solubles were partitioned with *n*-BuOH/H_2O. The *n*-BuOH solubles (10 g) were subjected to silica gel (350 g) column chromatography (Kieselgel, Merck) eluted with $CHCl_3$-MeOH (3 : 1, 200–300 ml) to give a pale yellow oil (80 mg). The fraction was separated by gel filtration [Sephadex L-20 (300 g); eluent, methanol (200–400 ml)] to give a 6-sulfo-*O*-α-D-quinovopyranosyldiacylglycerol (30 mg) as an amorphous solid; $[\alpha]_D^{27}$: +35.4° (*c* 1.0, $CHCl_3$/MeOH, 1 : 4); IR (film): ν = 3390, 1730, 1170, 1034 cm^{-1}; FAB-MS (negative): *m/z* = 765 $[M - Na]^+$; ^{1}H-NMR (600 MHz, $CDCl_3$/CD_3OD, 1 : 4): δ = 0.79 (6H, t, *J* = 7.0 Hz), 1.15–1.25 (48H, m), 1.52 (2H, tt, *J* = 14.5, 7.25 Hz), 1.53 (2H, tt, *J* = 14.5, 7.25 Hz), 2.24 (2H, t, *J* = 7.25 Hz), 2.26 (2H, t, *J* = 7.25 Hz), 2.94 (1H, dd, *J* = 14.5, 6.0 Hz), 3.13 (1H, dd, *J* = 10.0, 9.0 Hz), 3.24 (1H, dd, *J* = 14.5, 3.3 Hz), 3.36 (1H, dd, *J* = 10.0, 4.0 Hz), 3.51 (1H, dd, *J* = 10.0, 6.0 Hz), 3.55 (1H, dd, *J* = 10.0, 9.0 Hz), 3.94 (1H, m), 3.95 (1H, m), 4.10 (1H, dd, *J* = 12.0, 7.0 Hz), 4.37 (1H, dd, *J* = 12.0, 3.0 Hz), 4.72 (1H, d, *J* = 4.0 Hz) and 5.24 (1H, m); ^{13}C-NMR (150 MHz, $CDCl_3$/CD_3OD, 1 : 4): δ = 14.5 (2C, each q), 23.4 (2C, each t), 25.6 (t), 25.7 (t), 29.8–30.5 (18C, all t), 32.7 (2C, each t), 34.9 (t), 35.0 (t), 53.9 (t), 64.0 (t), 66.7 (t), 69.2 (d), 71.1 (d), 72.7 (d), 74.3 (d), 74.4 (d), 99.5 (d), 174.9 (s), 175.1 (s).

A solution of **1** (5 mg) in MeOH (0.5 ml) was treated with 3 % $NaOCH_3$/MeOH (0.5 ml) at room temperature for 20 min. The reaction mixture was neutralized with Dowex 50W × 8 (H^+ form) and partitioned with *n*-hexane/MeOH. Evaporation of the solvent at reduced pressure from the MeOH solubles yielded a 6-sulfo-*O*-α-D-quinovopyranosyl-(1→1′)-glycerol (2 mg) as an amorphous powder. The *n*-hexane solubles were evaporated at reduced pressure to give methyl palmitate, EI-MS; *m/z* = 270 $[M]^+$, and methyl myristate, EI-MS; *m/z* = 242 $[M]^+$, as a colorless oil.

Electron microscopic observation of Entomoneis *sp.*: The cells of *Entomoneis* sp. were suspended with hydrogen peroxide, and then cleaned by ultraviolet radiation followed by wash in distilled water. The specimen was dried on the sample stage and coated with gold-palladium using JEOL JFC-1100. The micrograph was taken by a JEOL F-15 scanning electron microscope.

Pharmacological tests: The method of the enzyme preparation and the reaction procedure were the same as described previously (12).

Bioassay of cytotoxic activity against P388 cell culture was performed by the method of Carmichael et al. (13).

Washed platelets were prepared by the method of Rho et al. and platelet aggregation was determined by a standard turbidometric method (14).

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Different Mechanisms Involved in the Vasorelaxant Effect of Flavonoids Isolated from *Satureja obovata*

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Abstract: **The inhibitory effects of naringenin, eriodictyol, and luteolin (10^{-5} and 5×10^{-5} M), previously isolated from *Satureja obovata* subsp. *obovata* var. *valentina* (Lamiaceae), on rat thoracic aorta were investigated. Flavonoids at the two concentrations assayed (10^{-5} and 5×10^{-5} M) showed different smooth muscle relaxant behaviour in the three phases involved in the noradrenaline (10^{-6} M)-induced contractions. The three flavonoids showed an inhibitory effect of the phasic component in order of potency: luteolin > eriodictyol > naringenin. Luteolin and eriodictyol inhibited both tonic-I and tonic-II phases associated to the inhibition of PKC and calcium influx, respectively, whereas naringenin only inhibited the tonic-I phase associated to inhibition of PKC.**

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At present, folk medicine is regarded as a very important source of new active principles. Some of them could be applied in therapy. *Satureja* species, well-known as Ajedrea, are aromatic plants belonging to the family Lamiaceae which grow in several areas of the Iberian peninsula. These plants have been used in traditional medicine as antimicrobial, spasmolytic, cicatrizant, and diuretic agents since antiquity. However, only the essential oil has been scientifically evaluated as spasmolytic agent (1, 2).

In previous works, we have shown the vascular and intestinal smooth muscle relaxant effects of the aqueous extract of two *Satureja obovata* Lag. varieties: var. *obovata* and var. *valentina*. Also we have shown that the spasmolytic effect of *Satureja obovata* subsp. *obovata* var. *valentina* aqueous extract was stronger than the variety *obovata* (3). According to a bioactivity-guided fractionation of the *Satureja obovata* subsp. *obovata* var. *valentina*, three active principles were isolated from the active extracts. These principles were identified by spectroscopic methods as flavonoids: luteolin, naringenin, and eriodictyol (4).

The aim of this work has been to evaluate the vascular smooth muscle relaxant effect of these flavonoids using a single bioassay which allowed recognition of the possible mechanism of action. We have selected the inhibition of the three components of the noradrenaline (10^{-6} M)-induced contraction in rat aorta: phasic component related to Ca^{2+} release from sarcoplasmic reticulum, tonic-I related to enzymatic systems as PKC, and tonic-II related to extracellular calcium influx (5).

Luteolin at two concentrations assayed (10^{-5} and 5×10^{-5} M), and eriodictyol and naringenin (5×10^{-5} M) exhibited an inhibitory effect on phasic contraction. Tonic-I phase was inhibited significantly by luteolin and eriodictyol, both at 10^{-5} and 5×10^{-5} M, and by naringenin (5×10^{-5} M). Finally tonic-II phase was affected only by luteolin and eriodictyol (10^{-5} and 5×10^{-5} M). Likewise, we have showed that nifedipine (10^{-7} M), a calcium channel blocker, inhibited the phasic and tonic-II phases, while H-7 (2×10^{-5} M), a PKC inhibitor, inhibited phasic and tonic-I components (Fig. **1**).

These results suggest that flavonoids exhibit an important vasorelaxant effect through different mechanisms: inhibition of the calcium release of the sarcoplasmic reticulum, inhibition of enzymatic systems as PKC and inhibition of the calcium influx which could be associated to the presence of one or two hydroxy substituents (3′ and 4′) in B ring.

However, the effect of these flavonoids on the noradrenaline-induced phasic contraction could be due to the inhibition of the PKC. In addition to calcium release of the sarcoplasmic reticulum, this enzyme has been involved in this phase (5) and we have observed that H-7, a PKC inhibitor, decreased the phasic component.

These results extend previous findings about the probable mechanism of action of flavonoids which were related to the inhibition of several enzymatic systems (PKC and others), which has been previously suggested (6, 7).