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A Study on Chemical Constituents of Three Red Algae of the Corallinaceae from Boso Peninsula in Japan

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Abstract Chemical constituents of three Corallinaceous algae *Corallina pilulifera* Postels et Ruprecht, *Amphiroa zonata* Yendo, and *Marginisporum crassissimum* (Yendo) Ganesan, collected at Tainoura, Amatsu-Kominato, Chiba, Japan, in May, 1999 were studied by thin-layer chromatography screening as well as antimicrobial activity assay against *Bacillus subtilis* (ATCC 6633). These three algae were revealed to have very similar TLC profiles to one another, and EtOAc layers of three algae showed weak antimicrobial activity. The ethyl acetate-soluble fraction of the methanol extract of *Corallina pilulifera* was revealed to contain pigments such as pyropheophorbide a, monoacylglycerol, unsaturated fatty acid, and sterols. The TLC assay of *n*-BuOH soluble fraction of *A. zonanta* (Amphiroideae) indicated unknown chemical substance lacking in the other two species of Corallinoideae.

Key words: Corallinaceae, *Corallina pilulifera*, *Amphiroa zonata*, *Marginisporum crassissimum*, Tainoura, Boso Peninsula, Japan, pyropheophorbide a, monoacylglycerol, Amphiroideae, Corallinoideae.

During our investigations on search for bioactive substances from marine and terrestrial natural resources, we initiated a collaborative program on chemical constituents of Corallinaceous algae collected around Chiba Prefecture. Here we describe a result of our recent study on the constituents of three Corallinaceous algae, *Corallina pilulifera* Postels et Ruprecht, *Amphiroa zonata* Yendo, and *Marginisporum crassissimum* (Yendo) Ganesan. Previously polysaccharides (Usov *et al.*, 1995) and glycerosphingolipids (Ishida *et al.*, 1993) were identified as chemical constituents of Corallinaceous algae such as *Corallina pilulifera*. Here we report the results of our recent studies on extracts of these red algae: 1) TLC (thin layer chromatography) assay and antimicrobial activity tests against *Bacillus subtilis* (ATCC 6633), and 2) chromatographical purification studies and spectroscopic analyses.

Materials and Methods

1. General Methods

^{1}H NMR (nuclear magnetic resonance) spectra were recorded on a JEOL JNM GSX-A400 and A500 spectrometers. Chemical shifts were expressed in δ value (ppm), and the 2.49 ppm and 7.26 resonances of residual dimethyl sulfoxide (DMSO) and chloroform, respectively, were used as an internal references in DMSO-d_6 and $CDCl_3$ solutions, respectively. TLC analyses were carried out by using precoated Keiselgel 60 F254 plates (Merck). Solvent systems were 1) hexane/EtOAc (2 : 1), 2) $CHCl_3$/MeOH (15 : 85), and 3) $CHCl_3$/*n*-BuOH/AcOH/H_2O (1.5 : 6 : 1 : 1). The spots were visualized by spraying reagent (10% Phosphomolybdic acid *n*-hydrate in MeOH). Silica gel PSQ 100B (Fuji Chemical Ltd.) and Sephadex LH-20 (Pharmacia) were used for glass column chromatographies. HPLC was performed on JASCO PU-980 apparatus with a reversed phase of semi-preparative column Develosil UG-5 (10×250 mm, Nomura Chemical Industries, Co. Ltd.). Solvent used as a mobile phase was 90% MeOH and flow rate was 2.0 mL/min. Detection was conducted at 220 nm using a JASCO UV detector (UV-970).

2. Algal Materials

Three red algae of the Corallinaceae, *Coral-*

lina pilulifera Postels et Ruprecht (Japanese name: Pirihiba), *Amphiroa zonata* Yendo (Japanese name: Usukawakaninote), and *Marginisporum crassissimum* (Yendo) Ganesan (Japanese name: Heritorikaninote) were collected at Tainoura, Amatsu-Kominato, Boso Peninsula, Japan, in May, 1999.

3. Extraction and Solvent Partition

(1) The alga *Corallina pilulifera* (600 g, wet weight) was extracted twice with MeOH (1.2 L and 0.8 L). The combined MeOH extract (18.0 g) was partitioned between EtOAc (400 mL×3) and H_2O (400 mL), and the aqueous phase was further extracted with *n*-BuOH (400 mL×3) to give EtOAc-layer (1.31 g), *n*-BuOH layer (2.59 g), and aqueous layer (10.62 g).

(2) The alga *Amphiroa zonata* (350 g, wet weight) was extracted twice with MeOH (1.0 L and 0.5 L). The combined MeOH extract (8.3 g) was partitioned between EtOAc (200 mL×3) and H_2O (200 mL), and the aqueous phase was further extracted with *n*-BuOH (200 mL×3) to give EtOAc-layer (0.81 g), *n*-BuOH layer (1.19 g), and aqueous layer (6.06 g).

(3) The alga *Marginisporum crassissimum* (100 g, wet weight) was extracted twice with MeOH (0.6 L and 0.5 L). The combined MeOH extract (3.3 g) was partitioned between EtOAc (100 mL×3) and H_2O (100 mL), and the aqueous phase was further extracted with *n*-BuOH (100 mL×3) to give EtOAc-layer (0.31 g), *n*-BuOH layer (0.49 g), and aqueous layer (2.34 g).

4. Purification of the EtOAc-layer of *C. pilulifera*

A part (1.09 g) of the EtOAc-soluble fraction (1.31 g) of *C. pilulifera* was subjected to silica gel column chromatography (3.0×30 cm) with gradient elution of MeOH in $CHCl_3$ to give 11 fractions (fr. 1A–1K).

The fraction 1E (61 mg), eluted with 2% MeOH in $CHCl_3$, was then separated by gel filtration on Sephadex LH-20 (1.5×53 cm; 100% MeOH) to give a fraction (1 mg) containing mainly pyropheophorbide a (Kobayashi, 1991): TLC, Rf values 0.21 (hexane/acetone, 1 : 1) and 0.59 ($MeOH/CHCl_3$, 15 : 85); ^1H NMR (DMSO-d_6) δ 9.71 (1H, s), 9.42 (1H, s), 8.87 (1H, s), 8.52 (1H, s), 8.20 (1H, brs), 6.36 (1 H, d), 6.19 (1H, d), 5.23 (1H, d), 5.08 (1H, d), 4.54 (1H, s), 4.28 (1H, s), 0.23 (1H, s), and −1.98 (1H, s).

The fraction 1F (57 mg), eluted with 2% MeOH in $CHCl_3$, was further purified by gel filtration on Sephadex LH-20 (1.5×60 cm; $CHCl_3$/MeOH, 3 : 1), followed by reversed-phase HPLC on ODS (Develosil UG-5; 10× 250 mm; 90% MeOH; flow rate, 2.0 mL/min; detection at 220 nm) to afford a monoacylglycerol (1 mg): ^1H NMR δ 5.37 (*ca.* 6H, m), 4.18 (2H, m), 3.93 (1H, m), 3.65 (2H, m), 2.84 (*ca.* 4H, m), 2.37 (2H, m), 2.12 (2H, m), 2.05 (2H, m), 1.30 (*ca.* 8-10H, m), and 0.89 (3H, t).

The fraction 1C (270 mg), eluted with 0–2% MeOH in $CHCl_3$, was separated by gel filtration on Sephadex LH-20 (1.4×62 cm; $CHCl_3$/MeOH, 2 : 1) followed by silica gel chromatography (1.0×10 cm; hexane/acetone, 19 : 1) to give a fraction (38 mg) mainly containing a sterol, which was implied to have a

Table 1. Results of the antimicrobial activity tests against *Bacillus subtilis*.

Sample*		Radius of blocking circle (mm)	Activity
Corallina pilulifera	EtOAc layer	1	+
	n-BuOH layer	0	−
	Aqueous layer	0	−
Amphiroa zonata	EtOAc layer	1	+
	n-BuOH layer	0	−
	Aqueous layer	0	−
Marginisporum crassissimum	EtOAc layer	1	+
	n-BuOH layer	0	−
	Aqueous layer	0	−

* Each sample was tested at 100 μg/disc.

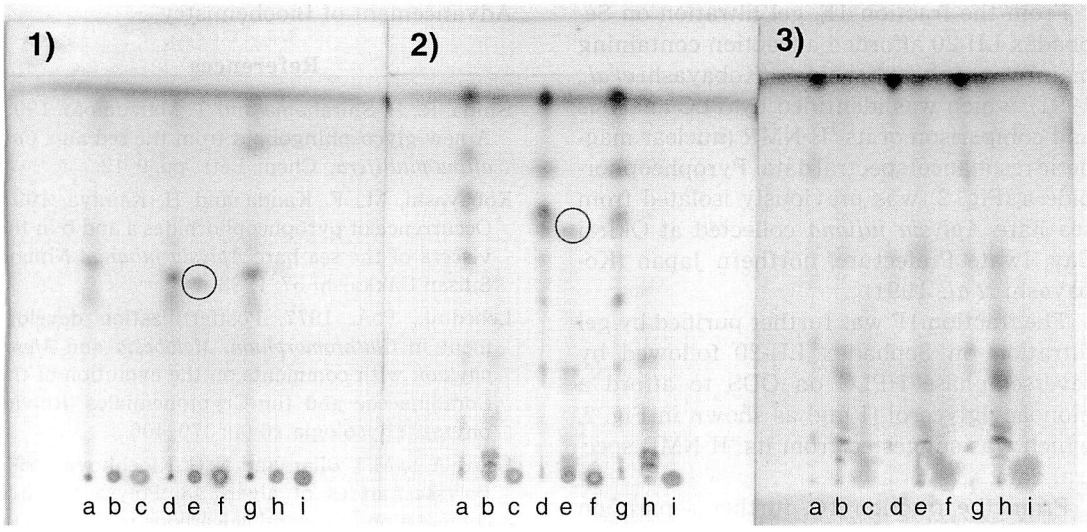
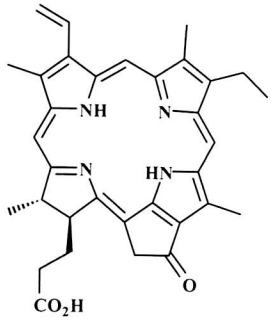
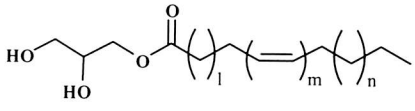


Fig. 1. Results of the TLC assay on silica gel plates.
Solvent systems: 1) hexane/EtOAc (2 : 1), 2) $CHCl_3$/MeOH (15 : 85), 3) $CHCl_3$/*n*-BuOH/AcOH/H_2O (1.5 : 6 : 1 : 1). Spray reagent: 10% Phosphomolybdic acid *n*-hydrate in MeOH. Lanes: a) *C. pilulifera*, EtOAc layer; b) *C. pilulifera*, *n*-BuOH layer; c) *C. pilulifera*, aqueous layer; d) *A. zonata*, EtOAc layer; e) *A. zonata*, *n*-BuOH layer; f) *A. zonata*, aqueous layer; g) *M. crassissimum*, EtOAc layer; h) *M. crassissimum*, *n*-BuOH layer; i) *M. crassissimum*, aqueous layer.



pyropheophorbide a



monoacylglycerol

Fig. 2. Structures of pyropheophorbide a and monoacylglycerol.

related structure to cholesterol on the basis of the TLC analysis [Rf values 0.54 (hexane/acetone, 2 : 1) and 0.37 (MeOH/$CHCl_3$, 1 : 99)] along with its ^{1}H NMR spectral data [δ 3.52 (1 H, m), 0.98 (3H, s) and 0.67 (3H, s)].

Results and Discussion

Three fractions (EtOAc layer, *n*-BuOH layer, and aqueous layer) from each three algae were subjected to TLC (thin layer chromatography) screenings (Fig. 1) on silica gel plate with three different solvent systems as well as antimicrobial activity tests against *Bacillus subtilis* (Table 1). As shown in Fig. 1, these three algae were revealed to have very similar TLC profiles to one aother, and EtOAc layers of three algae showed weak antimicrobial activity. From these results, we first chose the EtOAc layer of *Corallina pilulifera* for the study of chemical constituents.

The EtOAc-soluble fraction of the MeOH extract of *C. pilulifera* was subjected to a silica gel column chromatography (gradient elution of MeOH in $CHCl_3$) to give 11 fractions (fr. 1A–1K).

From the fraction 1E, gel filtration on Sephadex LH-20 afforded a fraction containing mainly pyropheophorbide a (Kobayashi *et al.*, 1991), which was identified by TLC analysis and comparison of its ^{1}H NMR (nuclear magnetic resonance) spectral data. Pyropheophorbide a (Fig. 2) was previously isolated from sea hare *Aplysia juliana* collected at Okirai Bay, Iwate Prefecture, northern Japan (Kobayashi *et al.*, 1991).

The fraction 1F was further purified by gel filtration on Sephadex LH-20 followed by reversed-phase HPLC on ODS to afford a monoacylglycerol (1 mg) as shown in Fig. 2, which was suggested from its ^{1}H NMR spectral data.

From the fraction 1C, further separation by gel filtration on Sephadex LH-20 followed by silica gel chromatography gave a fraction mainly containing a sterol, which was implied to have a related structure to cholesterol on the basis of the TLC analysis along with its ^{1}H NMR spectral data.

The *n*-BuOH-soluble fraction of *C. pilulifera* and EtOAc- and *n*-BuOH-soluble fractions of *A. zonata*, and *M. crassissimum* were also examined by the similar chromatographical procedures as described above to reveal that the constituents of these algal materials are similar to one another and unsaturated fatty acids as well as glycerolipids bearing sugar and unsaturated fatty acid moieties are predominantly contained. TLC and ^{1}H NMR screening showed that various tetrapyrrole-pigments like pyropheophorbide a were also contained in all three algae. However, it may be noteworthy that TLC assay results (Fig. 1) showed that the *n*-BuOH soluble fraction of *A. zonata* in the subfamily Amphiroideae contained a spot (indicated by circles in Fig. 1) that was not contained in *C. pilulifera* or *M. crassissimum* in the subfamily Corallinoideae (Lebednik, 1977).

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References

Ishida, R., H. Shirahama and T. Matsumoto. 1993. A new glycosphingolipid from the red alga *Corallina pilulifera*. Chem. Lett., pp. 9–12.

Kobayashi, M., F. Kanda and H. Kamiya. 1991. Occurrence of pyropheophorbides a and b in the viscera of the sea hare *Aplysia juliana*. Nippon Suisan Gakkaishi 57: 1983.

Lebednik, P. A. 1977. Postfertilization development in *Clathromorphum*, *Melobesia* and *Mesophyllum* with comments on the evolution of the Corallinaceae and the Cryptonemiales (Rhodophyta). Phycologia 16 (4): 379–406.

Usov, A. I., M. I. Bilan and N. G. Klochkova. 1995. Polysaccharides of algae. 48. Polysaccharaide composition of several calcareous red algae: isolation of alginate from *Corallina pilulifera* P. et R. Bot. Mar. 38: 43–51.

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房総半島産紅藻サンゴモ科3種の化学成分

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房総半島，天津小湊町鯛の浦において採取した紅藻サンゴモ科ピリヒバ (*Corallina pilulifera*), ウスカワカニノテ (*Amphiroa zonata*), ヘリトリカニノテ (*Marginisporum crassissimum*) の3種について，メタノール抽出後，溶媒分画を行い，酢酸エチル可溶部，ブタノール可溶部，および水可溶部を各々得た．これらについて，薄層クロマトグラフィーおよび枯草菌に対する抗菌活性試験によるスクリーニングを行った．その結果，各酢酸エチル可溶部に弱いながら抗菌活性が認められたので，ピリヒバの酢酸エチル可溶部を中心に化学成分分析を行った．各種クロマトグラフィーによる分離精製ならびに^{1}H NMR を中心とする機器分析の結果，本酢酸エチル可溶部には，pyropheophorbide a をはじめとする色素，モノアシルグリセロール，不飽和脂肪酸，ステロール等が主に含まれることが分かった．また，ブタノール可溶画分・TLC 分析の結果は，サンゴモ亜科に属すピリヒバとヘリトリカニノテには無い化学成分が，カニノテ亜科に属すウスカワカニノテに存在することを示唆した．