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An experimental approach to study the biosynthesis of brominated metabolites by the red algal genus *Laurencia*

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ABSTRACT

The production of labeled brominated metabolites with radioactive $^{82}$Br in Laurencia species was investigated as part of a study of the biosynthesis of halogenated metabolites from species belonging to the red algal genus Laurencia (Rhodomelaceae, Ceramiales). Radiobromide [$^{82}$Br], thin-layer chromatography (TLC), and TLC–autoradioluminography (ARLG) were used. When cultured in artificial seawater medium (ASP$_{12}$NTA including Na$^{82}$Br) under 16:8 h light:dark (LD) illumination cycles for 24 h, each of the strains of Laurencia, Laurencia japonensis Abe et Masuda, Laurencia nipponica Yamada (laurencin-producing race and laureatin-producing race), and Laurencia okamurae Yamada, produced species- (or race-) specific $^{82}$Br-containing metabolites. In the case of the laurencin-producing race of L. nipponica, laurencin and deacetyllaurencin were found to be produced in approximately 1:1 ratio, though laurencin is the major metabolite in the wild sample. Furthermore, when cultured in the dark, the production rates of brominated metabolites in Laurencia spp. were found to be diminished. The present study strongly indicates that the use of radiobromine [$^{82}$Br] in combination with the TLC-ARLG method is an effective approach for investigating the biosynthesis of brominated metabolites in Laurencia.
Keywords: *Laurencia* sp.; Rhodomelaceae; Brominated metabolites; Biosynthesis;

$^{82}$Br-compounds; TLC-ARLG method
1. Introduction

In the last four decades, various brominated organic compounds, which have not yet been encountered in other terrestrial organisms, have been discovered in marine organisms (Blunt et al., 2008). In particular, species of the red algal genus *Laurencia* (Rhodomelaceae, Ceramiales) are known to be prolific sources of a variety of halogenated secondary metabolites. *Laurencia* species produce bromine-containing compounds in far greater numbers than either chlorine- or iodine-containing ones. The Cl-containing compounds usually also possess Br atom(s), and only 4 I-containing metabolites have been found so far. To date, chemical compositions of more than 60 species of *Laurencia* throughout the world have been investigated. Around 500 diverse halogenated compounds, particularly sesquiterpenoids, diterpenoids, triterpenoids, and C$_{15}$ acetogenins, have been isolated from *Laurencia* (Erickson, 1983; Suzuki and Vairappan, 2005). Some of these halogenated compounds have been found to display antimicrobial, insecticidal, cytotoxic, and feeding-deterrent activities. Additional halogenated metabolites with a novel chemical structure still continue to be discovered from this unique genus (Blunt et al., 2008). However, studies of the biosynthesis of halogenated metabolites from *Laurencia* are rare, except for the enzymatic formation of
medium-sized cyclic bromo-ethers using lactoperoxidase (LPO) (Fukuzawa et al., 1990; Ishihara et al., 1997) or bromoperoxidase (BPO) that was partially purified from Laurencia nipponica Yamada (Fukuzawa et al., 1994).

The imaging plate (IP) is an established technology in autoradiography (Amemiya and Miyahara, 1988; Mori and Hamaoka, 1994). The thin-layer chromatography (TLC)-autoradioluminography (ARLG) method was developed using IPs to measure quantitatively the radioactivity ratio between the radiolabeled spots on a TLC plate (Motoji et al., 1995). We now report the application of the TLC-ARLG method to a biosynthetic study of brominated metabolites isolated from Laurencia. This paper deals with the production of various $^{82}$Br-compounds in the light or in the dark and discusses the usefulness of the TLC-ARLG method for the biosynthetic study on the brominated metabolites from Laurencia (Fig. 1).
Figure 1

from *Laurencia yaponensis*

1

2

3

4

5

from *Laurencia nipponica*

6: \( R = \text{Ac} \)

7: \( R = \text{H} \)

8

9

10: \( X = \text{H} \)

11: \( X = \text{Br} \)

12: \( R_1 = R_2 = \text{H} \)

13: \( R_1 = R_2 = \text{Ac} \)

14: \( R_1 = \text{H}, R_2 = \text{Ac} \)

from *Laurencia okamurae*

15

16

17

Suzuki et al.
Fig. 1. Molecular structures of brominated metabolites from *Laurencia* spp.
1; aplysiadiol, 2; japonenyne-A, 3; laurenenyne-A, 4; laurenenyne-B, 5; 2,10-dibromo-3-chloro-\(\alpha\)-chamigrene, 6; laurenycin, 7; deacetyllaurenycin, 8; laureatin, 9; isolaureatin, 10; laurenisol, 11; bromolaurenisol, 12; (3\(E\),6\(R\),7\(R\))-laurediol, 13; (3\(E\),6\(R\),7\(R\))-diacetyllaurediol, 14; (3\(E\),6\(R\),7\(R\))-6-acetyllaurediol, 15; laurinterol, 16; isolaurinterol, 17; \(\alpha\)-bromocuparene.

2. Results and discussion

\(\text{Na}^{82}\text{Br}\) was produced via neutron activation. Irradiation of natural abundance bromine atoms (ca. 1:1 mixture of \(\text{^{79}Br}\) and \(\text{^{81}Br}\)) with thermal neutrons in a nuclear reactor converts these stable isotopes into radioactive \(\text{^{80,82}Br}\) atoms, respectively. Although this process also activates stable sodium (\(\text{^{23}Na}\)) to \(\text{^{24}Na}\) (\(t_{1/2}\) 15.0 h), and a number of other trace elements present into their respective radionuclides, radioisotopically pure \(\text{^{82}Br}\), \(T_{1/2}\) 35.3 h, was recovered in relatively high radiochemical purity by allowing such short-lived radioactive contaminants in the irradiated samples to decay away over a 3-day cooling period.

Upon irradiation with thermal neutrons, the brominated C\(_{15}\) acetogenin, laurenycin (6) that had been isolated from *Laurencia nipponica* Yamada (as *Laurencia glandulifera* Kützing) (Irie et al., 1965, 1968c) showed 8 strong \(\gamma\)-rays in its gamma ray emission spectrum that are ascribable to \(\text{^{82}Br}\) (Murakami et al., 1982). The formation of
$^{82}\text{Br}$-lauренcin was confirmed by TLC and autoradiography (Figs. 2 and 3), which showed the radioactivated product (Lane A) and normal lauренcin (Lane B). The radioactivated lauренcin showed the same $R_f$ value on TLC as that of the normal lauренcin (Fig. 3). The (radio)chromatograms also show partial decomposition of lauренcin upon irradiation at the starting point (Fig. 2, Lane A), indicative of the expected de-(radio)debromination due to the Szilard-Chalmers effect (Szilard and Chalmers, 1934) and/or thermal decomposition through the irradiation.

All strains, *Laurencia japonensis* Abe et Masuda, *Laurencia nipponica* Yamada (lauренcin-producing race and laureat-in-producing race) (Masuda et al., 1997; Abe et al., 1999), and *Laurencia okamurae* Yamada produced the species- (or race-)specific $^{82}\text{Br}$-containing metabolites when cultured with Na$^{82}\text{Br}$, for example, the results from *L. japonensis* (Kamishima Island, Mie Prefecture) are shown in Figures 4 and 5. Both extracts exhibited similar spots, as shown in Fig. 5. The autoradiogram (Fig. 4, Lane C) displayed many radioactive bands, whose relative radioactivity is shown in Table 1. Some of the bands could be assigned to known brominated compounds by comparison of the $R_f$ values with those of the authentic samples. The rates of production of diterpene (aplysiadiol (1); major metabolite), sesquiterpene
(2,10-dibromo-3-chloro-α-chamigrene (5), and C₁₅ acetogenins (japonenyne-A (2) and
an inseparable mixture of laurenenyne-A (3) and -B (4)) (Fig. 1) approximately
reflected their yields in the wild sample (Takahashi et al., 1998, 1999; Suzuki et al.,
1993).

Production rates of brominated metabolites were examined at short intervals.
The results in the case of the laurencin-producing race of Laurencia nipponica are
shown in Fig. 6 and Table 2. As shown in Table 2, the rates of production of
halochamigrenes (Suzuki et al., 1979) and bromophenols (Suzuki and Kurosawa, 1979),
which are minor metabolites in the wild sample, were scarcely unchanged during 24
hours, but the rates of laurencin (6) and deacetyllaurencin (7) production were found to
have increased. Moreover, 6 and 7 were produced in approximately 1:1 ratio, although 6
is the major metabolite in the wild sample. On the other hand, in the case of the
laureatin-producing race, laureatin (8) and isolaureatin (9) were produced in
approximately 2:1 ratio (data not shown) as in the case of the wild sample (Irie et al.,
**Fig. 2.** Autoradiogram (TLC image recorded onto IP by BAS2000) for the radioactivated laurencin (6) (Lane A) and the normal laurencin (6) (Lane B).

**Fig. 3.** Thin-layer chromatogram for the radioactivated laurencin (6) (Lane A) and the normal laurencin (6) (Lane B).

**Fig. 4.** Autoradiogram (TLC image recorded onto IP by BAS2000) for the extract of *Laurencia japonensis* (Kamijima’s strain) cultured with Na$^{82}$Br for 24 h (Lane C) and that cultured with NaBr for 24 h (Lane D).

**Fig. 5.** Thin-layer chromatogram for the extract of *Laurencia japonensis* (Kamijima’s strain) cultured with Na$^{82}$Br for 24 h (Lane C) and that cultured with NaBr for 24 h (Lane D).

**Fig. 6.** Autoradiogram (TLC image recorded onto IP by BAS2000) for the extract of *Laurencia nipponica* (laurencin-producing race) cultured with Na$^{82}$Br for 4h (Lane E4), 8 h (Lane E8), 16 h (Lane E16) and 24 h (Lane E24), respectively.

**Fig. 7.** Autoradiogram (TLC image recorded onto IP by BAS2000) for the extract of *Laurencia japonensis* (Onahama’s strain) cultured with Na$^{82}$Br for 24 h under 16:8 h LD (Lane F) and that cultured with Na$^{82}$Br for 24 h in the dark (Lane G).

**Fig. 8.** Autoradiogram (TLC image recorded onto IP by BAS2000) for the extract of *Laurencia okamurae* cultured with Na$^{82}$Br for 24 h under 16:8 h LD (Lane H) and that cultured with Na$^{82}$Br for 24 h in the dark (Lane I).
The decrease of the producing rate of brominated metabolites was observed in all strains cultured with Na$^{82}$Br in the dark. In the case of *L. japonensis* (Onahama’s strain) (Fig. 7 and Table 3), the rates of production of aplysiadiol (1) and acetogenins, japonenyne-A (2) and an unidentified compound, decreased by approximately 75 percent, whereas the production of a small quantity of 2,10-dibromo-3-chloro-$\alpha$-chamigrene (5) was observed. On the other hand, in *L. okamurae* (Fig. 8 and Table 4), the rates of laurinterol (15), isolaurinterol (16), and $\alpha$-bromocuparene (17) (Irie et al., 1966, 1970b; Suzuki and Kurosawa, 1985) production decreased approximately by two thirds.

Table 1. Relative radioactivity for bands of the TLC, which were imaged on IP, of the extract from *Laurencia japonensis* (Kamijima’s strain) that was cultured in artificial seawater medium (ASP$_{12}$NTA including Na$^{82}$Br) at 15°C, 16:8 h LD for 24 h

<table>
<thead>
<tr>
<th>Band</th>
<th>Compound</th>
<th>PSL* (^{a)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>5</td>
<td>908.1</td>
</tr>
<tr>
<td>C-2</td>
<td>3 + 4</td>
<td>903.9</td>
</tr>
<tr>
<td>C-3</td>
<td>unidentified (^{b)} )</td>
<td>459.3</td>
</tr>
<tr>
<td>C-4</td>
<td>unidentified (^{c)} )</td>
<td>547.8</td>
</tr>
<tr>
<td>C-5</td>
<td>2</td>
<td>868.9</td>
</tr>
<tr>
<td>C-6</td>
<td>unidentified</td>
<td>771.2</td>
</tr>
<tr>
<td>C-7</td>
<td>1</td>
<td>1450.8</td>
</tr>
</tbody>
</table>

\(^{a)} \) BG; 1.4 PSL (mm$^{-2}$).

\(^{b)} \) A C$_{15}$ acetogenin.
A C<sub>15</sub> acetogenin.

Table 2. Relative radioactivity for bands of the TLC, which were imaged on IP, of the extracts from *Laurencia nipponica* (laurencin-producing race) that were cultured in artificial seawater medium (ASP<sub>12</sub>NTA including Na<sup>85</sup>Br) at 15°C, 16:8 h LD for 4 h, 8 h, 16 h and 24 h

<table>
<thead>
<tr>
<th>Band</th>
<th>Compound</th>
<th>PSL&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>E-1</td>
<td>halochamigrenes&lt;sup&gt;b)&lt;/sup&gt;</td>
<td>19.4</td>
</tr>
<tr>
<td>E-2</td>
<td>bromophenols&lt;sup&gt;c)&lt;/sup&gt;</td>
<td>28.1</td>
</tr>
<tr>
<td>E-3</td>
<td>6</td>
<td>39.2</td>
</tr>
<tr>
<td>E-4</td>
<td>7</td>
<td>17.8</td>
</tr>
</tbody>
</table>

<sup>a)</sup> BG; 1.4 PSL (mm<sup>-2</sup>).  
<sup>b)</sup> A mixture of halochamigrene derivatives, whose major component is 5.  
<sup>c)</sup> A mixture of bromophenols, containing 10 and 11.

Table 3. Relative radioactivity for bands of the TLC, which were imaged on IP, of the extracts from *Laurencia japonensis* (Onahama’s strain) that were cultured in artificial seawater medium (ASP<sub>12</sub>NTA including Na<sup>85</sup>Br) at 15°C for 24 h in the light (16:8 h LD) or in the dark

<table>
<thead>
<tr>
<th>Band</th>
<th>Compound</th>
<th>PSL&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L16D8</td>
</tr>
<tr>
<td>F-1, G-1</td>
<td>5</td>
<td>100.8</td>
</tr>
<tr>
<td>F-2, G-2</td>
<td>unidentified&lt;sup&gt;b)&lt;/sup&gt;</td>
<td>147.2</td>
</tr>
<tr>
<td>F-3, G-3</td>
<td>2</td>
<td>217.9</td>
</tr>
<tr>
<td>F-4, G-4</td>
<td>1</td>
<td>184.0</td>
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<sup>a)</sup> BG; 1.4 PSL (mm<sup>-2</sup>).  
<sup>b)</sup> A C<sub>15</sub> acetogenin.
Table 4. Relative radioactivity for bands of the TLC, which were imaged on IP, of the extracts from *Laurencia okamurae* that were cultured in artificial seawater medium (ASP$_{12}$NTA including Na$^{82}$Br) at $15^\circ$C for 24 h in the light (16:8 h LD) or in the dark.

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<th>Band</th>
<th>Compound</th>
<th>PSL*(a)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>L16D8</td>
</tr>
<tr>
<td>H-1, I-1</td>
<td><strong>17</strong></td>
<td>899.3</td>
</tr>
<tr>
<td>H-2, I-2</td>
<td><strong>16</strong></td>
<td>1021.3</td>
</tr>
<tr>
<td>H-3, I-3</td>
<td><strong>15</strong></td>
<td>1517.3</td>
</tr>
</tbody>
</table>

* BG; 1.7 PSL (mm$^{-2}$).

The *Laurencia* species, from which halogenated secondary metabolites have been found, possess *corps en cerise* in both superficial cortical cells and trichoblast cells. On the other hand, some *Laurencia* species that do not contain *corps en cerise* produce no halogenated metabolite (Suzuki and Vairappan 2005). *Corps en cerise*, an unusually swollen refractile inclusion, is recognized as the site of synthesis and/or storage of halogenated compounds (Young et al., 1980). Although these cell inclusions were found in 4-day-old plants in culture (Masuda et al., 1997), our preliminary examination showed that a brominated metabolite, epilaurallene (laurallene) was found to be produced in 24-hour-passed sporophytes after being released from *L. nipponica*.
(epilaurallene-producing race) (Suzuki et al., unpublished results). This suggests that the production of the halogenated metabolites starts at an early stage of the germination.

The size of *corps en cerise* must be small to be recognized during the first 3 days.

Brominated acetogenins and terpenoids, which have been isolated from various *Laurencia*, are thought to be synthesized via a different metabolic pathway and it is suggested that different sets of enzymes participate in their synthesis. Regarding to one of the relevant enzymes, vanadium-dependent bromoperoxidase (BPO) had been found in some green, brown, and red algae (e.g., *Ascophyllum nodosum* and *Corallina officinalis*) (Butler and Carter-Franklin, 2004), while the structure of *Laurencia*–derived BPO has not yet been characterized.

Fukuzawa and coworkers reported the enzymatic formation of medium-sized cyclic bromo-ethers found in *Laurencia* using lactoperoxidase (LPO) (Fukuzawa et al., 1990; Ishihara et al., 1997) or BPO (Fukuzawa et al., 1994), the latter of which was partially purified from *Laurencia nipponica*. They proposed without any proof that the bromine atom(s) in the brominated compounds derived from marine organisms could be introduced via bromo cationic species that is generated by the two-electron oxidation of bromide ion with BPO and hydrogen peroxide. Any biosynthetic work on halogenated
metabolites from *Laurencia* has not yet been reported so far besides the afore-mentioned work.

When cultured with Na$^{82}$Br in the artificial seawater medium (ASP$_{12}$NTA) under 18:6 h LD for 24 h, each of the strains of *Laurencia* spp. produced the species- (or race-)specific $^{82}$Br-containing metabolites. However, it is noteworthy that the laurencin-producing race of *L. nipponica* produced laurencin (6) and deacetyllaurencin (7) in approximately 1:1 ratio (Fig. 6 and Table 2), although the TLC of Lane E24 (data not shown) revealed that the band of 6 was far larger than that of 7, reflecting the yields of 6 and 7 in the wild sample. Biogenetically (Kikuchi et al., 1991) laurencin (6) has been presumed to be produced from (3$E$,6$R$,7$R$)-laurediol (12) (Fig. 1), which has been found from *Laurencia nipponica* (Fukuzawa et al., 1972), via intramolecular bromonium ion-catalyzed cyclization followed by enzymatic acetylation. Deacetyllaurencin (7) was really synthesized from 12 with LPO (Fukuzawa et al., 1990) or BPO (Fukuzawa et al., 1994) in the presence of hydrogen peroxide (H$_2$O$_2$) and sodium bromide (NaBr). Fukuzawa et al. (1972) separated (3$E$,6$R$,7$R$)-laurediol (12) and (3$E$,6$R$,7$R$)-diacetylaurediols (13) as an inseparable mixture of optical isomers with (6$R$,7$R$) and (6$S$,7$S$) and geometrical isomers with 3$E$ and 3$Z$ from the
laureatin-producing race of *L. nipponica*.

Our present results suggested the presence of \((3E,6R,7R)\)-6-acetyllaurediol (14), which may afford laurencin by intramolecular bromonium ion-catalyzed cyclization, in the laurencin-producing race of *L. nipponica*. Since the resulting deacetyllaurencin would then enzymatically be acetylated, laurencin would be accumulated in larger quantities than deacetyllaurencin during a long period.

In addition, when cultured in the dark, the decrease of the producing rate of brominated metabolites in all strains of *Laurencia* spp. strongly suggested that the secondary metabolism of brominated C15 acetogenins and terpenoids in *Laurencia* depends upon light condition.

The autoradiograms of the extracts from all *Laurencia* strains examined exhibited several unidentified bands. This suggests that some biogenetic intermediates with a short life were produced. The isolation and identification of these metabolites may provide important information for biogenesis of *Laurencia* metabolites.

Results of the present study strongly indicate that the TLC-ARLG method can be utilized for \(^{82}\text{Br}\)-labelled compounds and is a useful tool for the biosynthetic study of brominated metabolites from *Laurencia*, though the half-life of \(^{82}\text{Br}\) atom is very short.
(35.3 h) as compared with those of the more commonly used tracers $^{14}$C atom (5730 y)
and $^{3}$H atom (12.3 y).

In conclusion, when cultured in the artificial seawater medium (ASP$_{12}$NTA
including Na$^{82}$Br) under 18:6 h LD for 24 h, each of the strains of Laurencia spp.,

Laurencia japonensis Abe et Masuda, Laurencia nipponica Yamada

(laurencin-producing race and laureatin-producing race), and Laurencia okamurae

Yamada, produced the expected species- (or race-)specific $^{82}$Br-containing metabolites.
3. Experimental

3.1. Radioactivation of brominated compounds

Laurencin (6) (ca. 10 mg; ~ 2.3 mg Br) was sealed in a 1 cm square polyethylene sheet and packed in a polyethylene capsule. The capsule was irradiated for one min under a thermal neutron flux of $4.0 \times 10^{13}$ n/cm$^2$/sec (TA irradiation pipe) in the JRR-4 (or JRR-3) reactor of the Japan Atomic Energy Research Institute (JAERI) (the present Japan Atomic Energy Agency (JAEA)). Natural abundance sodium bromide ($^{23}$Na$^{79/81}$Br) (ca. 100 mg; ~ 38 mg $^{81}$Br) was also irradiated as in the case of the radioactivation of laurencin.

3.2. Radionuclide analysis of radioactive compounds

Gamma-ray measurement of irradiated products was made after a cooling interval of 3 days to eliminate contributions to the gamma-ray spectrum by $^{80/80m}$Br ($T_{1/2}$ 17.7 min, $^{24}$Na ($T_{1/2}$ 15.0 h), and other short-lived neutron activation radionuclides. The gamma analysis was performed with a p-type detector (GEX15190P; 15.6% relative efficiency, and 1.85 keV resolution at the 1332 keV $^{60}$Co peak (SEIKO EG&G ORTEC Co. Ltd.).
3.3. Identification of $^{82}$Br-compounds by the Imaging plate-TLC method

Solutions of the radioactivated and authentic laurencin were spotted on a silica gel thin-layer (TLC) aluminum sheet (Merck, Kieselgel 60F$_{254}$), and the TLC sheet was developed with toluene/AcOEt (4:1) as a developing solvent. When the solvent front reached the top of the TLC sheet, it was removed and air-dried. For autoradiography, the TLC sheet was covered with polyethylene film and placed on an imaging plate (IP; Fuji Photo Film Co., Ltd.), placed into a cassette, and the cassette was closed and allowed to stand for 3 days in the dark inside a lead shield. The IP was then processed using the BAS-2000 system (Fuji Photo Film Co., Ltd.). Bands of the TLC sheet imaged on the IP were visually discriminated (e.g., Fig. 2). The PSL value (photo-stimulated luminescence; relative intensity of radiation of bands) of each of the bands was determined according to the standards for effective values by Fuji Photo Film Co., Ltd. A random area B excluding bands on the same TLC sheet was encircled. The PSL value per 1 mm$^2$ in a random area B was defined as background (BG). The relative intensity of radiation of a band A is defined as the following equation:

$$PSL_A^* (\text{mm}^{-2}) = PSL_A \times \frac{1}{A_A} - BG = PSL_B \times \frac{A_A}{A_B} \times \frac{1}{A_A}$$

where $PSL_A$ = the PSL value of a band A, $A_A$ = the area (mm$^2$) of a band A, $PSL_B$ = the
PSL value of a random area B, and $A_B = \text{the area (mm}^2\text{)}$ of a random area B.

After completing the IP analysis, the TLC sheet was sprayed with 5% phosphomolybdic acid in ethanol and heated at 80–100°C to visualize the colorless substances as blue-colored bands (e.g., Fig. 3).

3.4. Algal samples

The strains of Laurencia japonensis Abe et Masuda (collected at Kamishima Island, Mie Prefecture and at Onahama, Fukushima Prefecture), Laurencia nipponica Yamada (laurencin-producing race) (collected at Tanesashi, Aomori Prefecture), L. nipponica (laureatin-producing race) (collected at Moheji, Hokkaido), and Laurencia okamurae Yamada (collected at Inubosaki, Chiba Prefecture), which had been subcultivated at the Laboratory of Algal Systematics, Division of Biological Sciences, Graduate School of Science, Hokkaido University, were used.

3.5. Culture

One individual of each of the strains in a single dish (71 mm x 61 mm) containing 150 ml artificial seawater medium (ASP$_{12}$NTA including radioactivated NaBr (1.5 mg)) was placed in a plant-growth chamber illuminated with cool-white
fluorescent lamps (3000 lux) at 15°C for 24 h under 16:8 h light:dark (LD) illumination cycles, or at 15°C for 24 h in the dark. Furthermore, *Laurencia nipponica* (laurencin-producing race) was cultured at 15°C for 4 h, 8 h, 16 h, and 24 h, respectively, under 16:8 h LD. In addition, as a reference culture, one individual of each of the strains was also cultured under the same conditions using normal NaBr.

3.6. Extraction, thin-layer chromatography, exposure on imaging plate, and visualization with phosphomolybdic acid

Each of the cultured algae was blotted with a paper towel and immersed in MeOH for 2 h. The MeOH was decanted and the MeOH solution was concentrated under reduced pressure. The residue was then partitioned between Et₂O and H₂O. The Et₂O solution was concentrated under reduced pressure to leave an oily material.

As in the case of laurencin, solutions of each of the extracts (300 μg) were spotted on a TLC sheet, and each of the TLC sheets was developed with toluene, exposed on an IP for 3 days followed by autographic analysis, and visualized with 5% phosphomolybdic acid as described.
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