HETEROCYCLES, Vol. 96, No. 2, 2018, pp. 273 - 285. © 2018 The Japan Institute of Heterocyclic Chemistry
Received, 13th December, 2017, Accepted, 30th December, 2017, Published online, 19th January, 2018
DOI: 10.3987/COM-17-13854

COUMARINS WITH ANTI-MELANOGENESIS ACTIVITIES FROM A CHEMICALLY ENGINEERED EXTRACT OF A MARINE-DERIVED FUNGUS

Hitoshi Kamauchi, Kaoru Kinoshita, and Kiyotaka Koyama*

Department of Pharmacognosy and Phytochemistry Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose-shi, Tokyo 204-8588, Japan.
E-mail: kiyotaka@my-pharm.ac.jp (Kiyotaka Koyama)

Abstract – Chemical investigation of new resources is critical for developing medicinal leads. Chemically engineered extracts, a technique for derivatization of natural extracts, was performed on crude extracts of marine-derived fungus *Eurotium rubrum*; the resulting reaction mixture exhibited enhanced tyrosinase inhibition. Coumarin derivatives (**1-3**) were isolated from this chemically engineered extract. The structures of **1-3** were established using NMR, MS, and IR methods, revealing coumarin structures with side chains biosynthesized by the acetate-malonate pathway. **1-3** showed tyrosinase inhibition (IC_{50} = 7.7, 13.3, and 9.5 μM, respectively) and anti-melanogenesis activity (IC_{50} = 6.6, 7.2, and 6.6 μM, respectively, against B16 melanoma cells).

INTRODUCTION

Natural compounds have high diversity in their structures and exhibit various bioactivities, though some natural compounds are difficult to synthesize. Therefore a large number of drugs have been developed from natural resources.[1]

However Convention on Biological Diversity (CBD) established a general framework for access and benefit-sharing associated with the use of genetic resources.[2] The international community has deemed that the discovery and development of novel and biologically active compounds from natural sources should be guided by the CBD principles; these guidelines are expected to facilitate productive interactions between biodiversity-rich source countries and science and technology-rich countries.[3]

Taking advantage of existing resources is also important. OSMAC[4] and chemical epigenetics[5] have been used to induce increased structural diversity of secondary metabolites, leading to the discovery of various

new compounds. Structural derivatization of natural compounds *via* diversity-oriented synthesis[6] has proven to be an attractive approach for producing new compounds.[7] In fact, natural compound derivatives have been developed based on various combinations of chemical-synthetic and biosynthetic methods. The natural compound derivatives generated by modification of natural compounds were expected to show novel bioactivities.

Chemically engineered extracts (CEE) was developed as a novel way to obtain such novel natural product derivatives. CEE comprises the directed chemical diversification of natural extracts and provides an opportunity for generating compounds with interesting bioactive properties.[8] For instance, the CEE of an *n*-butanol extract of the plant *Polygonum ferrugineum* Wed (Polygonaceae) by treatment with hydrazine monohydrate yielded new pyrazole derivatives that showed antifungal activity.[9]

Comparison of CEE with the modern method, derivatization of isolated natural compounds, suggests that there are advantages to the CEE method, including the following, (i) The targets of derivatization are all compounds contained in the natural extract, permitting the simultaneous generation of multiple compounds. (ii) Evaluation of bioactivity for the CEE product permits assessment of the effectiveness of the derivatization before complicated isolation schemes are attempted. On the other hand, the modern method requires schemes for the isolation of natural compounds in order to prepare starting materials; evaluation of bioactivity has to await derivatization of individual compounds.

The aldehyde group is a prime target for derivatization. This moiety has high reactivity and readily provides a new C-C bond, as exemplified by the Grignard and Wittig reactions. Knoevenagel condensation is considered one of the easiest ways to convert an aldehyde group to a new C-C bonds. This condensation consists of a nucleophilic addition of an activated methylene to an aldehyde group, thereby affording a new olefin.[10] The introduction of new C-C bond linkages is an effective approach for structural derivatization of natural compounds. These linkages can provide unnatural scaffolds by a combination of natural compounds and chemical reagents.

Extracts of marine-derived fungi are attractive resources. Fungal metabolites with unique structures may be associated with a variety of biological activities.[11] Given that fungi are able to adapt to various environments, there is much diversity in their metabolisms.[12]

RESULTS AND DISCUSSION

The marine-derived fungus *Eurotium rubrum* is a rich source of compounds with aldehyde groups, as indicated by the presence of CH carbons at around 200 ppm in the ^{13}C-NMR spectrum of the crude $CHCl_3$ extracts of *E. rubrum* (Figure S1). Notably, our previous study seeking natural products with anti-melanogenesis activity identified diketopiperazine compounds as the active compounds,[13] but that work did not yield the isolation of compounds with aldehyde groups.

Knoevenagel condensation was performed on a crude $CHCl_3$ extract of *E. rubrum*. The CEE was shown to possess a stronger tyrosinase inhibitory activity than the natural crude extract (Table 1). The HPLC profile of the CEE indicated that the concentrations of some compounds were decreased while some new peaks were observed when compared to the crude extract (Figure 1). These new peaks were expected to represent the enhanced tyrosinase inhibitory activity. After scale-up derivatization, the CEE was subjected to silica-gel column chromatography (CC), Sephadex LH-20 CC, octadecylsilyl silica-gel CC, and HPLC. Three new coumarin derivatives (**1**-**3**) were isolated (Figure 2).

Table 1. Tyrosinase inhibitory activities of chemically engineered extract performed on the *E. rubrum* $CHCl_3$ extract, and those of the *E. rubrum* $CHCl_3$ extract itself

Inhibition of tyrosinase activity (%, mean ± SD, n=3)				
	Conc. (μg/mL)			
	0.78	1.56	3.12	6.25
chemically engineered extract	5.0±0.5**	9.1±0.9**	20.8±4.4**	31.1±6.1**
E. rubrum $CHCl_3$ ext.	−0.5±0.8	−0.9±3.6	0.0±2.5	6.0±1.9

*, P <0.05; **, P <0.01 vs. *E. rubrum* $CHCl_3$ ext.

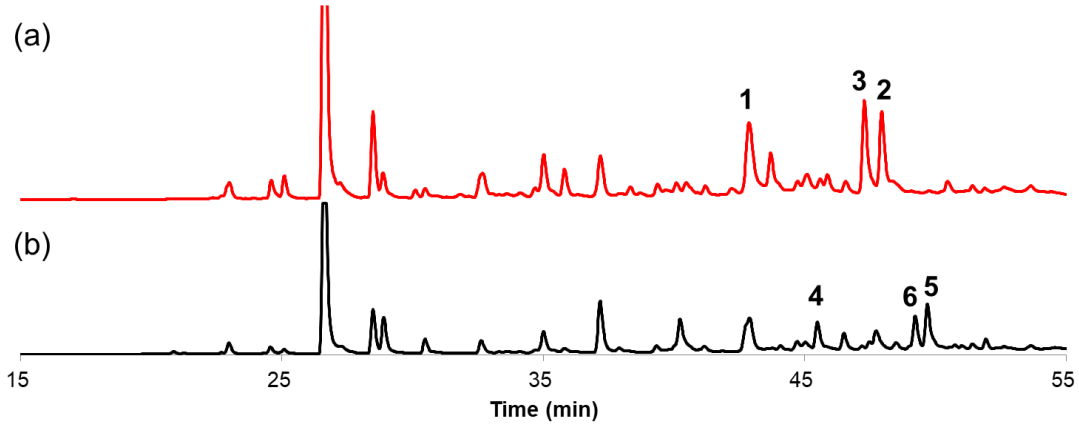


Figure 1. HPLC profile of (a) chemically engineered extract performed on the *E. rubrum* $CHCl_3$ extract and that of (b) the *E. rubrum* $CHCl_3$ extract itself

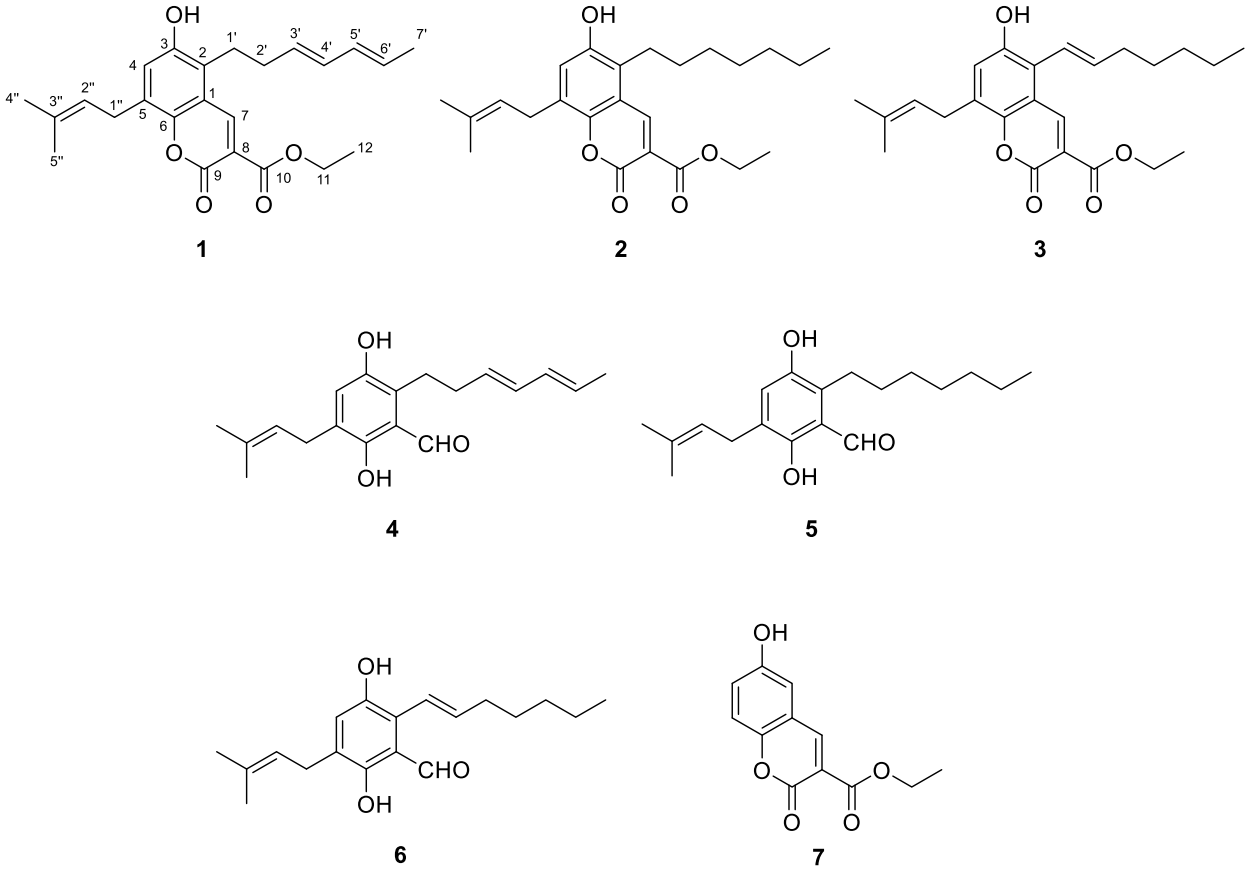


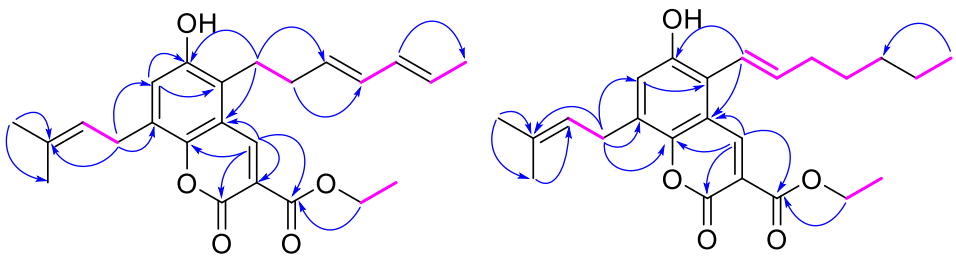
Figure 2. Structures of new coumarin derivatives (**1**-**3**), known hydroquinone compounds (**4**-**6**) and synthesized coumarin (**7**)

Compound **1** was obtained as yellow oil and the molecular formula was deduced to be $C_{24}H_{28}O_5$ by HREIMS (*m/z* 396.1936 $[M]^+$). The IR spectrum showed absorptions attributable to hydroxy (3316 cm^{-1}) and carbonyl (1749 and 1717 cm^{-1}) groups. The ^{1}H-NMR spectrum showed signals attributable to the following: two aromatic protons at δ_H 7.03 (s, H-4) and δ_H 8.68 (s, H-7); five olefinic protons at δ_H 5.58 (dt, *J*=13.5, 7.6 Hz, H-3'), δ_H 6.00 (t, *J*=13.5 Hz, H-4'), δ_H 5.97 (t, *J*=13.5 Hz, H-5'), δ_H 5.59 (dq, *J*=13.5, 6.6 Hz, H-6') and δ_H 5.27 (t, *J*=7.3 Hz, H-2''); four methylenes (one oxygenated) at δ_H 4.42 (q, *J*=7.1 Hz, H-11), δ_H 2.95 (t, *J*=7.6 Hz, H-1'), δ_H 2.33 (q, *J*=7.6 Hz, H-2') and δ_H 3.47 (d, *J*=7.3 Hz, H-1''); and four methyl protons at δ_H 1.41 (t, *J*=7.1 Hz, H-12), δ_H 1.73 (d, *J*=6.6 Hz, H-7'), δ_H 1.73 (s, H-4''), and δ_H 1.72 (s, H-5'') (Table 2). Furthermore, the ^{13}C-NMR spectrum showed the following: two carboxyl groups at δ_C 157.1 (C-9) and δ_C 163.8 (C-10); fourteen olefinic carbons at δ_C 117.0 (C-1), δ_C 123.4 (C-2), δ_C 149.8 (C-3), δ_C 122.7 (C-4), δ_C 128.4 (C-5), δ_C 148.0 (C-6), δ_C 146.2 (C-7), δ_C 117.1 (C-8), δ_C 129.5 (C-3'), δ_C 132.0 (C-4'), δ_C 131.0 (C-5'), δ_C 128.2 (C-6'), δ_C 120.3 (C-2'') and δ_C 134.6 (C-3''); four methylene carbons at δ_C 61.9 (C-11), δ_C 25.3 (C-1'), δ_C 33.2 (C-2'), and δ_C 27.3 (C-1''); and four methyl carbons at δ_C 14.2 (C-12), δ_C 17.9 (C-7'), δ_C 25.8 (C-4'') and δ_C 18.0 (C-5'') (Table 2). These spectroscopic data for

1 were similar to those for the known prenyl polyketide isodihydroauroglaucin (**4**).[14] However the aldehyde proton and carbon at C-7 and hydroxyl proton at C-6 of **4** were not present in the spectra for **1**. Instead of these protons and carbons, new protons and carbons for **1** were observed (C-8 to C-12). Key HMBC correlations were present from H-7 to C-1, C-6, C-8, C-9, and C-10, and from H-11 to C-10; additional ^{1}H-^{1}H COSY correlations were evident from H-11 to H-12 (Figure 3). These correlations suggested that **1** possessed a coumarin structure with an ethyl ester at C-8. The *trans*-configurations of the side chain were confirmed by observation of the coupling constants of four olefinic protons at H-3' to H-6'.

Compound **2** possessed the molecular formula $C_{24}H_{32}O_5$, as deduced by HRFABMS (*m/z* 401.2325 $[M+H]^+$). ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **1** (Table 2), indicating that the structure of **2** was similar to that of **1**. Signals for the olefinic protons and carbons of the side chain of **1** were not observed; methylene protons and carbons (δ_H 1.26, 1.28 and 1.35 and δ_C 22.7, 29.1, 29.5 and 31.8 at C-3' to C-6') were seen instead. These spectroscopic data indicated that **2** had the same structure as **1** without olefins at side chain (Figure 2).

Compound **3** possessed the molecular formula $C_{24}H_{30}O_5$, as deduced by HRFABMS (*m/z* 399.2169 $[M+H]^+$). ^{1}H- and ^{13}C-NMR spectra of **3** were similar to those of **1** and **2** (Table 2). Spectroscopic data for **3** indicated the presence of an olefin (δ_H 6.45, d, J=16.3, δ_C 120.2 and δ_H 6.09, dt, J=16.3, 7.0, δ_C 142.6). HMBC correlations were detected from H-1' to C-1 and C-3. These correlations suggested the presence of an olefin at C-1' and C-2' (Figure 3). The *trans*-configuration of C-1' to C-2' was determined by the coupling constant of H-1' and H-2'.



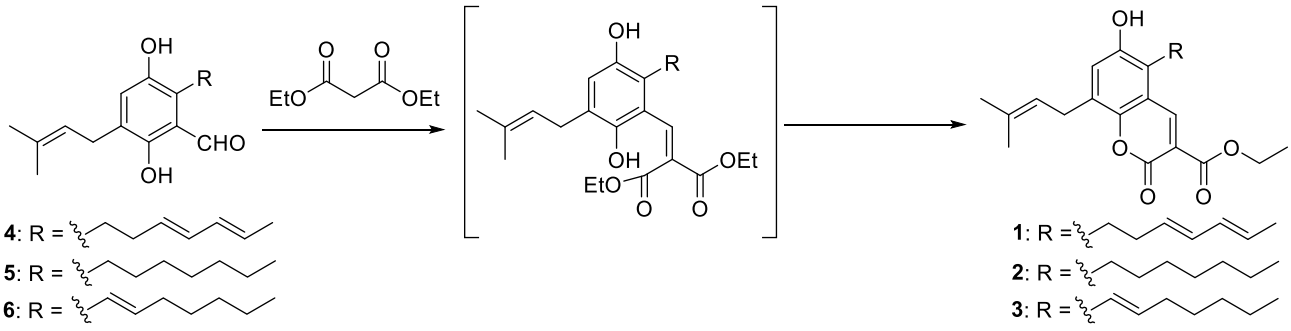
1 **3**

Figure 3. 2D-NMR (^{1}H-^{1}H COSY: pink bold lines, HMBC: blue arrows) of coumarin derivatives (**1** and **3**)

Table 2. ^{1}H- and ^{13}C-NMR spectroscopic data for **1-3** (400 MHz for ^{1}H, 100 MHz for ^{13}C in $CDCl_3$)

	1		**2**		**3**	
Pos.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	117.0		117.2		116.3	
2	123.4		124.6		120.2	
3	149.8		149.8		149.1	
4	122.7	7.03 (s)	122.6	7.01 (s)	122.4	7.09 (s)
5	128.4		128.1		129.8	
6	148.0		148.1		148.0	
7	146.2	8.68 (s)	146.2	8.71 (s)	146.1	8.65 (s)
8	117.1		117.0		117.4	
9	157.1		157.1		156.8	
10	163.8		163.9		163.8	
11	61.9	4.42 (q, 7.1)	62.0	4.43 (q, 7.2)	61.9	4.41 (q, 7.2)
12	14.2	1.41 (t, 7.1)	14.2	1.42 (t, 7.2)	14.2	1.40 (t, 7.2)
1'	25.3	2.95 (t, 7.6)	25.1	2.85 (t, 7.7)	120.2	6.45 (d, 16.3)
2'	33.2	2.33 (q, 7.6)	30.8	1.56 (m)	142.6	6.09 (dt, 16.3, 7.0)
3'	129.5	5.58 (dt, 13.5, 7.6)	29.1	1.35 (overlapped)	33.6	2.36 (q, 7.0)
4'	132.0	6.00 (t, 13.5)	29.5	1.35 (overlapped)	28.8	1.54 (m)
5'	131.0	5.97 (t, 13.5)	31.8	1.26 (overlapped)	31.5	1.33 (overlapped)
6'	128.2	5.59 (dq, 13.5, 6.6)	22.7	1.28 (overlapped)	22.5	1.34 (overlapped)
7'	17.9	1.73 (d, 6.6)	14.1	0.88 (t, 6.4)	14.0	0.93 (t, 7.0)
1"	27.3	3.47 (d, 7.3)	27.3	3.47 (d, 7.6)	27.5	3.50 (d, 7.5)
2"	120.3	5.27 (t, 7.3)	120.4	5.27 (t, 7.6)	120.2	5.29 (t, 7.5)
3"	134.6		134.5		134.8	
4"	25.8	1.73 (s)	25.8	1.73 (s)	25.8	1.75 (s)
5"	18.0	1.72 (s)	17.9	1.72 (s)	17.9	1.73 (s)

The structures and HPLC data suggested that the isolated compounds (**1**-**3**) were not observed in the crude extract and were instead produced by the CEE method. Plausible synthetic pathways were proposed based on the assumption that the main components of the *E. rubrum* crude extract (prenyl hydroquinone compounds isodihydroauroglaucin (**4**),[15] flavoglaucin (**5**)[15] and tetrahydroauroglaucin (**6**)[14] respectively) served as the starting materials, given that these peaks were not observed in the HPLC profile of the CEE (Figure 1). Isolated coumarin derivatives would then be produced from the hydroquinone compounds *via* Knoevenagel condensation at C-7 to C-8 and condensation at C-6-OH to C-9-CO_2Et (Scheme 1).



Scheme 1. Plausible synthetic pathway of **1-3**

The newly isolated compounds (**1-3**), the hydroquinone compounds (**4-6**), and synthesized coumarin without a side chain and prenyl group (**7**)[16] were tested for inhibitory activity of tyrosinase, the key enzyme of melanogenesis. All of the coumarin derivatives showed inhibitory activities against tyrosinase (Table 3). As above, coumarin without a side chain and prenyl group (**7**) did not exhibit detectable inhibition of tyrosinase. The compounds were further assessed for inhibitory activity against melanin synthesis using theophylline-stimulated B16 melanoma 4A5 cells. **1-3** showed inhibitory activities that were stronger than the original hydroquinone compounds (**4-6**) (Table 3). In contrast, coumarin without a side chain and prenyl group (**7**) did not show anti-melanogenesis activity.

Table 3. Melanogenesis inhibitory activities of compounds **1-7**

Comp.	Tyrosinase activity (IC_{50}, μM)	Melanogenesis activity (IC_{50}, μM)
1	7.7	6.6
2	13.3	7.2
3	9.5	6.6
4	31.3	51.2
5	21.7	46.6
6	9.9	>100
7	>100	>100
kojic acid	30.3	n.d.
α-arbutin	n.d.	608

n.d. no data.

CEE treatment of a natural crude extract of *E. rubrum* enhanced tyrosinase inhibitory activities. Three new coumarin derivatives (**1-3**) were isolated from the CEE; each showed tyrosinase inhibitory and anti-melanogenesis activities. The difference between the tyrosinase inhibitory activity of the CEE and that of the crude extract was explained by the observation that the activities of **1** and **2** were stronger than those of the respective original compounds.

Though the tyrosinase inhibitory activity of **3** was unchanged compared to that of the respective original compound, the anti-melanogenesis activity of **3** was stronger than that of **6**. These results suggested that coumarin derivatization influenced not only inhibition of tyrosinase activity but also the activity of other components of the melanogenesis cascade.

Isolated coumarin derivatives possessing side chains biosynthesized by the acetate-malonate pathway are rarely present in natural coumarins, because most natural coumarins are biosynthesized *via* the shikimate pathway. Therefore, the unnatural structures (coumarin skeletons with polyketide side chains) observed in the CEE presumably reflected the combination of biosynthesis and chemical synthesis. Moreover, the presence of side chains and a prenyl group appeared to be critical to the anti-melanogenesis activities, given that a coumarin compound without side chains (7) did not show any anti-melanogenesis activities. In summary, new anti-melanogenesis compounds with unnatural structures were obtained by the CEE method. Our results show that the CEE method constitutes a valuable novel approach for the discovery of medicinal resources.

EXPERIMENTAL

General experimental procedures

IR spectra were recorded with a Thermo FT-IR Nicolet iS5 spectrophotometer (ATR, Thermo Fisher Scientific, Waltham, MA). UV spectra were recorded with a Thermo GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific). ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-AL400 MHz spectrometer (JEOL, Tokyo, Japan), using tetramethylsilane as the internal standard. Low- and high-resolution FAB and EIMS spectra were measured with a JEOL JMS-700 spectrometer (JEOL). Column chromatography was performed using silica gel 60N (63-210 μm, Kanto Chemical, Tokyo, Japan); ODS silica gel YMC-GEL ODS-A (YMC, Kyoto, Japan); and Sephadex LH-20 (GE Healthcare, Buckinghamshire, UK). Preparative HPLC was performed on a SSC-3461 (2.5 mL/min, Senshu Scientific Tokyo, Japan), equipped with a SSC-5410 UV detector at 254 nm (Senshu Scientific) with an Inertsustain C_{18} column (10 ϕ × 250 mm, GL Sciences, Tokyo, Japan), and a Jasco PU-2080 Plus (2.5 mL/min, Jasco, Tokyo, Japan) equipped with a Jasco PU-2075 Plus UV detector at 254 nm (Jasco) with a Senshu Pak PEGASIL Silica SP-100 column (10 ϕ × 250 mm, Senshu Scientific).

Fungal material

The marine-derived fungus *E. rubrum* (MPUC136) was isolated from seaweed obtained in Chosei-mura, Chosei-gun, Chiba Prefecture, Japan, in December, 2005. The isolate was speciated by rDNA sequence analysis. The internal transcribed spacer regions 1 and 2 and the 5.8S rDNA in the rRNA gene of the

isolate were identical to those of an epitype strain of *E. rubrum*. A voucher specimen (MPUC136) was deposited at the department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University.

Fermentation and extraction

E. rubrum was pre-incubated on PGY (peptone: Kyokuto Pharmaceutical Industrial, Tokyo, Japan; glucose: Iwaki Seiyaku Tokyo, Japan; yeast extract: Becton, Dickinson and Company, Franklin Lakes, NJ) slant medium. After pre-incubation, *E. rubrum* was inoculated into 500-mL Roux flasks (16 flasks) containing wheat (Hakubaku, Yamanashi, Japan, 150 g per flask) immersed in artificial sea water (Instant Ocean, NAPQO, Concord, Ohio). Flasks were statically incubated at 26 °C in the dark for 14 days. Fermented substrate was extracted with $CHCl_3$.

Coumarin derivatization

Small-scale derivatization: *E. rubrum* $CHCl_3$ extract (100 mg) and diethyl malonate (20 μL, Tokyo Chemical Industry, Tokyo, Japan) were substituted with piperidine (50 μL; Wako Pure Chemical Industries, Osaka, Japan) in EtOH (1 mL). The solution was stirred under reflux for 15 min and then concentrated *in vacuo* to yield a coumarin derivatization reaction mixture. This reaction mixture was used for each anti-melanogenesis assay.

Large-scale derivatization: *E. rubrum* $CHCl_3$ extract (2.0 g) and diethyl malonate (4 mL) were substituted with piperidine (1 mL) in EtOH (10 mL). The solution was stirred under reflux for 15 min and then concentrated *in vacuo* to yield a coumarin-derivatized reaction mixture. The components of this reaction mixture were shown to match those of the small-scale derivatization. This reaction mixture was used for isolation of the components.

Analytical HPLC conditions

Analytical HPLC was performed on a Jasco PU-2089 Plus (1.0 mL/min, Jasco) equipped with a Jasco UV-2075 Plus UV detector at 254 nm (Jasco) with a CAPCELL PAK C_{18} MGII column (4.6 ϕ × 250 mm, OSAKA SODA, Osaka, Japan). HPLC profiles of the $CHCl_3$ extract of *E. rubrum* and the coumarin-derivatized reaction mixture are provided in Figure 1. Solvent conditions were MeCN and H_2O (0-10 min: 20:80, 10-50 min: from 20:80 to 100:0, 50-60 min: 100:0), and flow rates were 1.0 mL/min. The *E. rubrum* $CHCl_3$ extract was resolved at a concentration of 1 mg/mL (10 μL charged). The coumarin-derivatized reaction mixture was resolved at a concentration of 4 mg/mL (10 μL charged). Following purification, the isolated compounds were each resolved at concentrations of 1 mg/mL (10 μL charged).

*Isolation of coumarin compounds (**1-3**)*

The coumarin derivatized reaction mixture (5.7 g) was fractionated by silica-gel column chromatography with *n*-hexane/EtOAc (2:1) to yield four fractions (a-d). Fraction c (529.0 mg) was subjected to two chromatography steps: (1) Sephadex LH-20 column chromatography with MeOH, and (2) ODS column chromatography with MeCN/H_2O (8:2), yielding **1** (13.4 mg) from fraction c1.

Fraction b (3.8 g) was subjected to two chromatography steps: (1) silica-gel column chromatography with *n*-hexane/acetone (10:1, 7:1, 1:1), and (2) preparative HPLC with *n*-hexane/acetone (4:1), yielded two fractions (b1 and b2). **2** (7.1 mg, t_R 11 min) was obtained as fraction b1. **3** (1.5 mg) was obtained from fraction b2 by preparative HPLC with MeCN/H_2O (8:2, t_R 20 min).

Compound **1**: Yellow oil; UV (MeOH) λ_{max} (log ε): 217 (4.81), 318 (4.35), 391 (3.75); IR ν_{max} (ATR): 3316, 1749, 1717 cm^{-1}; EIMS (pos.) *m/z* (rel. int., %): 396 $[M]^+$ (48) 323 (100), 315 (56), 267 (30); HREIMS (pos.) *m/z* 396.1936 $[M]^+$ (calculated for $C_{24}H_{28}O_5$, 396.1937); ^{1}H- and ^{13}C-NMR, see Table 2.

Compound **2**: Yellow oil; UV (MeOH) λ_{max} (log ε): 213 (4.50), 319 (4.17), 389 (3.47); IR ν_{max} (ATR): 3401, 1755, 1721 cm^{-1}; FABMS (pos.) *m/z*: 401 $[M+H]^+$ 355; HRFABMS (pos.) *m/z* 401.2325 $[M+H]^+$ (calculated for $C_{24}H_{33}O_5$, 401.2328); ^{1}H- and ^{13}C-NMR, see Table 2.

Compound **3**: Yellow oil; UV (MeOH) λ_{max} (log ε): 219 (4.01), 325 (3.77), 392 (3.22); IR ν_{max} (ATR): 3373, 1741 cm^{-1}; FABMS (pos.) *m/z*: 399 $[M+H]^+$ 353; HRFABMS (pos.) *m/z* 399.2169 $[M+H]^+$ (calculated for $C_{24}H_{31}O_5$, 399.2171); ^{1}H- and ^{13}C-NMR, see Table 2.

*Isolation of hydroquinone compounds (**4-6**)*

E. rubrum $CHCl_3$ extract (23.0 g) was dissolved with EtOAc. The EtOAc-soluble portion (20.2 g) was fractionated by silica-gel column chromatography with $CHCl_3$-MeOH (100:1, 50:1, 25:1, 10:1, 5:1, 0:100) to yield four fractions (a'-d'). Fraction a (3.5 g) was subjected to silica-gel column chromatography with *n*-hexane-acetone (4:1) to yield three fractions (a'a'-a'c'). **4** was obtained as fraction a'c' (673.5 mg). Fraction a'a' (774.0 mg) was subjected to silica-gel column chromatography with *n*-hexane-$CHCl_3$ (1:2) yielded **5** (406.2 mg) and **6** (219.7 mg).

Synthesis of 7

2,5-Dihydroxybenzaldehyde (100 mg, 0.72 mmol; Sigma-Aldrich, St. Louis, MO) and diethyl malonate (900 μL, 5.96 mmol) were dissolved with piperidine (100 μL) in EtOH (1.5 mL). The solution was stirred at reflux for 15 min and then concentrated *in vacuo*. The reaction mixture was distributed with by EtOAc

and H_2O. The organic layer was dried over $MgSO_4$ and purified by silica-gel column chromatography (*n*-hexane-EtOAc 2:1 to 1:1), yielding **7**[16] (78.3 mg, 0.33 mmol, 46%).

Tyrosinase inhibition assay

Tyrosinase inhibitory activity was assayed as described previously with slight modifications,[17] using a 96-well clear polystyrene microplate (Corning, Corning, NY). Each of the test compounds was dissolved in DMSO. The final DMSO concentration was 10%; validation experiments demonstrated that this concentration of DMSO did not interfere with the detection of dopachrome. Mushroom tyrosinase (T3824, Sigma-Aldrich) was formulated in phosphate buffer (0.1 mol/L phosphate buffer, pH 6.8; Wako Pure Chemical Industries). L-DOPA (Sigma-Aldrich) was dissolved in phosphate buffer immediately before use in the assay. Phosphate buffer (100 μL) was combined with mushroom tyrosinase (30 μL of a dilution sufficient to yield a final assay concentration of 1.44 U/well) and DMSO-dissolved test sample (20 μL). Freshly prepared L-DOPA (50 μL) was added to yield a final assay concentration of 625 μM. After incubation for 15 min at room temperature, dopachrome formation was measured at an absorbance (Abs) at 475 nm using an Immuno Mini NJ-2300 microplate reader (BIOTEC, Tokyo, Japan). DMSO without test compound was used as the negative control; kojic acid (Tokyo Chemical Industry) was used as a positive control. The inhibitory activity was calculated as follows: % inhibition = $[(Abs_{control}-Abs_{sample})/Abs_{control}]\times100$. The IC_{50} values were estimated using Prism software (version 5.02; GraphPad, San Diego, CA).

Cell culture

B16 melanoma 4A5 cells (RCB 0557) were purchased from RIKEN Cell Bank and were cultured using Dulbecco's Modified Eagle's medium (DMEM: Wako Pure Chemical Industries) with 10% fetal bovine serum (FBS: Wako Pure Chemical Industries) at 37 °C in 5% CO_2.

Melanin synthesis inhibitory assay

The melanin content was determined by a slightly modified method.[18] Each compounds were dissolved in DMSO. The final DMSO concentration in the medium was 0.1%.

The assay was performed in 6-well clear polystyrene microplates (Corning). B16 melanoma cells were seeded into each well as 9.6×10^4 cells in a 2-mL volume. After incubation for 24 h at 37 °C in 5% CO_2, test compounds and theophylline (to a final concentration in the medium of 1 mM; Sigma-Aldrich) were added. After incubation for 72 h, cells were washed with phosphate-buffered saline (PBS; Wako Pure Chemical Industries) and harvested using 0.25% trypsin. The cell suspension was first washed with PBS,

and then the cells were counted using a Scepter™ 2.0 Handheld Automated Cell Counter (Merck Darmstadt, Germany). Cells were solubilized in 1 mL of 1 N NaOH for 60 min at 80 °C. Lysates were transferred to a 96-well microplate (Corning, Inc.). Absorbance was measured at 475 nm (reference wavelength: 650 nm) using an Immuno Mini NJ-2300 (BIOTEC Tokyo, Japan) microplate reader; melanin concentrations were extrapolated from a standard curve of known concentrations of synthetic melanin (Sigma-Aldrich). Alpha-arbutin (Glico, Osaka, Japan) was used as a positive control. The IC_{50} values were estimated using Prism software (version 5.02; GraphPad, San Diego, CA). The cytotoxicity (cell number <85%) was not appeared around the concentration of IC_{50} values.

ACKNOWLEDGEMENTS

This study was supported by a Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan.

REFERENCES

1. D. J. Newman and G. M. Cragg, *J. Nat. Prod.*, 2016, **79**, 629.
2. L. N. Joppa, P. Visconti, C. N. Jenkins, and S. L. Pimm, *Science*, 2013, **341**, 1100.
3. G. M. Cragg, F. Katz, D. J. Newman, and J. Rosenthal, *Nat. Prod. Rep.*, 2012, **29**, 1407.
4. R. T. Hewage, T. Aree, C. Mahidol, S. Ruchirawat, and P. Kittakoop, *Phytochemistry*, 2014, **108**, 87.
5. T. Asai, T. Yamamoto, and Y. Oshima, *Tetrahedron Lett.*, 2011, **52**, 7042.
6. S. L. Schreiber, *Science*, 2000, **287**, 1964.
7. T. Asai, K. Tsukada, S. Ise, N. Shirata, M. Hashimoto, I. Fujii, K. Gomi, K. Nakagawara, E. N. Kodama, and Y. Oshima, *Nat. Chem.*, 2015, **7**, 737.
8. I. A. Ramallo, M. O. Salazar, L. Mendez, and R. L. E. Furlan, *Acc. Chem. Res.*, 2011, **44**, 241.
9. S. N. López, I. A. Ramallo, M. G. Sierra, S. A. Zacchino, and R. L. E. Furlan, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 441.
10. Z. Zhang, B. Xiao, Q. Chen, and X.-Y. Lian, *J. Nat. Prod.*, 2010, **73**, 252.
11. J. F. Sanchez, A. Somoza, N. P. Keller, and C. C. C. Wang, *Nat. Prod. Rep.*, 2012, **29**, 351.
12. G. Strobel, B. Daisy, U. Castillo, and J. Harper, *J. Nat. Prod.*, 2004, **67**, 257.
13. H. Kamauchi, K. Kinoshita, T. Sugita, and K. Koyama, *Bioorg. Med. Chem. Lett.*, 2016, **26**, 4911.
14. T. Hamasaki, Y. Kimura, Y. Hatsuda, and M. Nagao, *Agric. Biol. Chem.*, 1981, **45**, 313.
15. K. Yoshihira, C. Takahashi, S. Sekita, and S. Natori, *Chem. Pharm. Bull.*, 1972, **20**, 2727.
16. F. J. Martínez-Martínez, R. S. Razo-Hernández, A. L. Peraza-Campos, M. Villanueva-García, M. T. Sumaya-Martínez, D. J. Cano, and Z. Gómez-Sandoval, *Molecules*, 2012, **17**, 14882.

17. M. A. Peralta, M. G. Ortega, A. M. Agnese, and J. L. Cabrera, *J. Nat. Prod.*, 2011, **74**, 158.
18. M. Miyake, S. Yamamoto, O. Sano, M. Fujii, K. Kohno, S. Ushio, K. Iwaki, and S. Fukuda, *Biosci. Biotechnol. Biochem.*, 2010, **74**, 753.