Bioorganic & Medicinal Chemistry 21 (2013) 779–787

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Alkaloid constituents from flower buds and leaves of sacred lotus (*Nelumbo nucifera*, Nymphaeaceae) with melanogenesis inhibitory activity in B16 melanoma cells ☆

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ARTICLE INFO

Article history:
Received 24 September 2012
Revised 12 November 2012
Accepted 16 November 2012
Available online 5 December 2012

Keywords:
Nelumbo nucifera
Melanogenesis inhibitor
N-Methylasimilobine *N*-oxide
Aporphine-type alkaloid
Benzylisoquinoline-type alkaloid
Medicinal flower

ABSTRACT

Methanolic extracts from the flower buds and leaves of sacred lotus (*Nelumbo nucifera*, Nymphaeaceae) were found to show inhibitory effects on melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells. From the methanolic extracts, a new alkaloid, *N*-methylasimilobine *N*-oxide, was isolated together with eleven benzylisoquinoline alkaloids. The absolute stereostructure of the new alkaloid was determined from chemical and physicochemical evidence. Among the constituents isolated, nuciferine, *N*-methylasimilobine, (−)-lirinidine, and 2-hydroxy-1-methoxy-6a,7-dehydroaporphine showed potent inhibition of melanogenesis. Comparison of the inhibitory activities of synthetic related alkaloids facilitated characterization of the structure-activity relationships of aporphine- and benzylisoquinoline-type alkaloids. In addition, 3–30 μM nuciferine and *N*-methylasimilobine inhibited the expression of tyrosinase mRNA, 3–30 μM *N*-methylasimilobine inhibited the expression of TRP-1 mRNA, and 10–30 μM nuciferine inhibited the expression of TRP-2 mRNA.

1. Introduction

The Nymphaeaceae plant *Nelumbo* (*N*) *nucifera* Gaertn. is widely distributed throughout Asia and Oceania and has many common names (e.g., sacred lotus, Indian lotus, Chinese water lily). Since ancient times, all parts of this plant have been used not only as a vegetable and food garnish but also as a traditional medicine. For example, the flower buds of this plant are used both to treat blood vomiting, bleeding due to internal and external injury and various skin diseases, and as a sedative and an anti-inflammatory in traditional Chinese medicine. The flower buds are also used in Indian Ayurvedic medicine for the treatment of premature ejaculation, abdominal cramps and bloody discharge and as a cardiac tonic. The leaves of *N. nucifera* are used to treat hematemesis, epistaxis, hemoptysis, hematuria, hyperlipidemia, and obesity. As chemical constituents of the flower buds of *N. nucifera*, many flavonol glycosides have been isolated from the stamens, while the leaves are reported to be rich in a number of alkaloids together with flavonol glycosides.[2] However, detailed chemical and pharmacological studies on the bioactive constituents of the flower buds and leaves of *N. nucifera* have yet to be reported.

We have been searching for inhibitors of melanin production from natural medicines to develop new compounds for cosmetic products and depigmenting agents.[3–5] Recently, we reported the isolation and structure elucidation of melanogenesis inhibitors from the flower buds of *Camellia japonica*.[6,7] As a continuation of studies on melanogenesis inhibitors from medicinal flowers,[8–18] we found that methanolic (MeOH) extracts and alkaloid fractions from the flower buds and leaves of *N. nucifera* showed inhibitory effects on melanogenesis.

In this paper, we describe the isolation of aporphine- and benzylisoquinoline-type alkaloids with melanogenesis inhibitory activity from the flower buds and leaves of *N. nucifera*, as well as elucidation of the structure of a new alkaloid, *N*-methylasimilobine *N*-oxide. In addition, the inhibitory effects of related commercial and synthesized isoquinoline-type alkaloids on melanogenesis were examined and their structure-activity relationships were characterized. Lastly, the principal alkaloid constituents with melanogenesis inhibitory activity were examined for their effects on the expression of tyrosinase and TRP-1 and TRP-2 mRNA.

☆ This paper is number 37 in our series "Medicinal Flower". For paper number 36, see Ref. 1.

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http://dx.doi.org/10.1016/j.bmc.2012.11.038

2. Results and discussion

2.1. Isolation of melanogenesis inhibitors from the flower buds and leaves of *N. nucifera*

Melanocytes can be stimulated by many effectors including ultraviolet radiation[19] and α-melanocyte-stimulating hormone (α-MSH).[20] It is generally accepted that the cAMP pathway plays a key role in the regulation of melanogenesis and that cAMP is involved in α-MSH-stimulated signal transduction.[21,22] In the present study, we used a phosphodiesterase inhibitor, theophylline, to stimulate B16 melanoma 4A5 cells. As shown in Table 1, the MeOH extract from the flower buds of *N. nucifera* cultivated in Thailand significantly inhibited melanogenesis with an IC_{50} value of 20 μg/mL in theophylline-stimulated murine B16 melanoma 4A5 cells. The MeOH extract of the leaves exhibited a moderate effect, whereas the inhibitory activity of the stamens and seeds was weak. The extracts from the flower buds, stamens, and seeds showed no cytotoxic effects and that of the leaves showed weak cytotoxicity at a high concentration of 100 μg/mL (Table 1).

The MeOH extracts prepared under reflux from the flower buds and leaves were suspended in H_2O, and then extracted with ethyl acetate (EtOAc) followed by 1-butanol (1-BuOH). The EtOAc- and 1-BuOH-soluble fractions from the flower buds strongly inhibited melanogenesis with IC_{50} values of 9.5 and 10.1 μg/mL, respectively. The 1-BuOH-soluble fraction from the leaves also showed potent inhibition (IC_{50} 34.6 μg/mL). These fractions showed weak cytotoxic effects at a high concentration (Table 2).

The EtOAc- and 1-BuOH-soluble fractions from the flower buds and leaves were subjected to ordinary- and reversed-phase silica gel column chromatography, followed by HPLC to yield a new alkaloid termed *N*-methylasimilobine *N*-oxide (**1**),[**] together with nuciferine (**2**),[*,**,23-25] nuciferine *N*-oxide (**3**),[**,26,27] *N*-nornuciferine (**4**),[*,**,25,28] *N*-methylasimilobine (**5**),[*,**,24,29] asimilobine (**6**),[**,30] (−)-lirinidine (**7**, 5-demethylnuciferine),[*,**,31,32] dehydronuciferine (**8**),[**,33,34] 2-hydroxy-1-methoxy-6a,7-dehydroaporphine (**9**),[**,34] lysicamine (**10**),[*,**,35] D,L-armepavine (**11**),[**,36] pronuciferine (**12**),[*,**,37] kaempferol 3-*O*-β-D-glucopyranoside,[*,38] quercetin 3-*O*-β-D-glucopyranoside,[**,39] isorhamnetin 3-*O*-β-D-glucopyranoside,[*,40] quercetin 3-*O*-β-D-galactpyranoside,[**,41] (+)-catechine,[**] β-sitsterol,[*] and palmitic acid[*] (*, from flower buds; **, from leaves) (Fig. 1). The flower buds of *N. nucifera* were extracted with MeOH at room temperature to give a MeOH extract (rt), which was partitioned with a diethyl ether (Et_2O)/1% aqueous acetic acid (AcOH) mixture to yield an Et_2O fraction and aqueous AcOH phase. The aqueous AcOH phase was made basic with 10% NH_4OH solution, and then subjected to extraction with Et_2O. The Et_2O-soluble fraction was washed with 1% aqueous NaOH to yield an alkaloid fraction. The alkaloid fraction from the flower buds showed potent inhibition of melanogenesis with an IC_{50} value of 4.2 μg/mL. Using a procedure to that described above, **2**, **4**, **5**, **7**, **10**, and **12** were isolated from the alkaloid fraction (Table 3).

2.2. Structure of *N*-methylasimilobine *N*-oxide (1)

N-Metylasimilobine oxide (**1**) was obtained as a pale yellow oil with negative optical rotation ($[\alpha]_D^{24}$ −56.3°). The IR spectrum of **1** showed absorption bands at 3400 and 1509 cm^{-1} ascribable to hydroxyl and aromatic groups. In the EI-MS spectrum of **1**, a molecular ion peak was observed at *m*/*z* 297 (M^+) and the molecular formula of **1** was determined to be $C_{18}H_{19}NO_3$ by high-resolution (HR) MS measurement. The ^1H (CD_3OD) and ^{13}C NMR (Table 4) spectra of **1**, which were assigned by various NMR experiments,[42] showed signals due to two methyls, [δ 3.39 (O←N-CH_3), 3.56 (OCH_3)], three methylenes, a methane, and five aromatic protons. The proton and carbon signals in the ^1H and ^{13}C NMR data of **1** were superimposable on those of *N*-methylasimilobine (**5**), except for those around the *N*-oxide, whereas the proton and carbon signals in the *N*-oxide part were similar to those of nuciferine *N*-oxide (**3**).[26] The relative stereostructure of the *N*-oxide region of **1** was clarified by NOESY experiment, which showed a NOE correlation between the 6a-proton and the *N*-methyl group. Next, oxidation of *N*-methylasimilobine (**5**) with *m*-chloroperbenzoic acid (*m*-CPBA)[26] yielded **1**. In addition, O-methylation of **5** with trimethysilyldiazomethane ($TMSCH_2N_2$) yielded nuciferine (**2**), whose absolute configuration has been determined by synthesis.[25] Consequently, the absolute configuration of the 6a position in **1** and **5** was elucidated to be *R*. On the basis of these findings, the absolute stereostructure of **1** was determined to be as shown in Figure 2.

2.3. Effects of alkaloids constituents (2–12) and related alkaloids (19a, 19b, 23) on melanogenesis

Among the constituents isolated from the flower buds and leaves of *N. nucifera*, the N-methylated aporphine-type alkaloids nuciferine (**2**), *N*-methylasimilobine (**5**), (−)-lirinidine (**7**) and 2-hydroxy-1-methoxy-6a,7-dehydroaporphine (**9**) significantly inhibited melanogenesis with IC_{50} values of 15.8, 14.5, 19.3, and 13.3 μM, respectively, whereas dehydronuciferine (**8**; IC_{50} ca. 85 μM),[43] and aporphine-type alkaloids lacking the *N*-methyl group, namely *N*-nornuciferine (**4**), asimilobine (**6**) and lysicamine (**10**), did not show such potent inhibitory activity. The N-methylated benzylisoquinoline-type alkaloid, D,L-armepavine (**11**), also showed activity equipotent to that of the N-methylated aporphine-type alkaloids. On the other hand, nuciferine *N*-oxide (**3**) and an N-methylated proaporphine-type alkaloid, pronuciferine (**12**), showed weak inhibitory activity. In addition, two alkaloids (**7**, **10**) were observed to have cytotoxic effects at a high concentration (100 μM) (Table 5). These results indicate that the *N*-methyl group is essential for potent inhibitory activity, while the *N*-oxide group markedly reduces this activity.

Next, to obtain further information on the structural requirements for potent inhibition of melanogenesis, the effects of related commercial (**13**, **14**) and synthesized isoquinoline-type alkaloids

Table 1
Inhibitory effects of the MeOH extracts from several parts of *N. nucifera* on melanogenesis in B16 melanoma 4A5 cells

Concn (μg/mL)	Inhibition (%)						IC_{50} (μg/mL)
	Control	1	3	10	30	100	
Flower buds[a]	0.0 ± 2.7	14.3 ± 1.2**	16.5 ± 2.4**	29.8 ± 3.2**	62.1 ± 1.6**	91.7 ± 1.8**	20.3
Stamen[a]	0.0 ± 3.6	8.0 ± 3.2	1.5 ± 4.1	6.0 ± 4.4	8.8 ± 5.1	12.9 ± 2.3**	–
Seeds[a]	0.0 ± 2.5	−5.3 ± 2.3	−0.3 ± 2.9	9.9 ± 7.4	14.7 ± 2.7	18.0 ± 2.3*	–
Leaves[b]	0.0 ± 2.7	1.3 ± 2.5	6.8 ± 1.8	10.3 ± 1.0**	12.1 ± 1.7**	45.2 ± 1.1**	–

Each value represents the mean ± S.E.M., (n = 4).
Significantly different from the control group, *p < 0.05, **p < 0.01.
[a] The cell viabilities for the MeOH extracts from the flower buds, stamen, and seeds at 100 μM are more than 96%.
[b] The cell viability for the MeOH extract from the leaves at 100 μM is 86.5%.

Table 2
Inhibitory effects of the EtOAc-, 1-BuOH- and H_2O-soluble fractions from the flower buds and leaves of *N. nucifera* on melanogenesis in B16 melanoma 4A5 Cells

Concn (μg/mL)	Inhibition (%)						IC_{50} (μg/mL)
	control	1	3	10	30	100	
Flower buds							
EtOAc fraction[a,b]	0.0 ± 3.3	4.0 ± 6.4	26.9 ± 4.4**	46.9 ± 1.6**	81.8 ± 2.5**	93.3 ± 3.0**	9.5
1-BuOH fraction[a,c]	0.0 ± 5.5	13.4 ± 3.1	−7.2 ± 11.4	48.2 ± 1.1**	75.7 ± 1.1**	73.5 ± 2.7**	10.1
H_2O fraction[a,b]	0.0 ± 4.2	13.8 ± 3.0	14.7 ± 2.2	12.6 ± 2.0**	18.0 ± 1.4**	22.9 ± 0.3**	—
Leaves							
EtOAc fraction[a,b]	0.0 ± 4.0	11.5 ± 3.8	13.5 ± 3.6	14.0 ± 0.7	2.9 ± 22.1	11.4 ± 1.6	—
1-BuOH fraction[a,b]	0.0 ± 2.6	5.0 ± 3.7	12.7 ± 4.5*	24.1 ± 1.1**	42.8 ± 3.9**	83.5 ± 0.4**	34.6
H_2O fraction[a,b]	0.0 ± 2.5	12.5 ± 3.6*	17.3 ± 2.6**	29.7 ± 1.7**	27.6 ± 1.9**	46.2 ± 3.2**	—

Each value represents the mean ± S.E.M., (n = 4).
Significantly different from the control group, *p < 0.05, **p < 0.01.
[a] The cell viabilities for all fractions at 30 μM are more than 88%.
[b] The cell viabilities for the EtOAc and H_2O fractions from flower buds and the EtOAc, 1-BuOH, and H_2O fractions from leaves at 100 μM are more than 78%.
[c] The cell viability for the 1-BuOH fraction from flower buds at 100 μM is 36.9%.

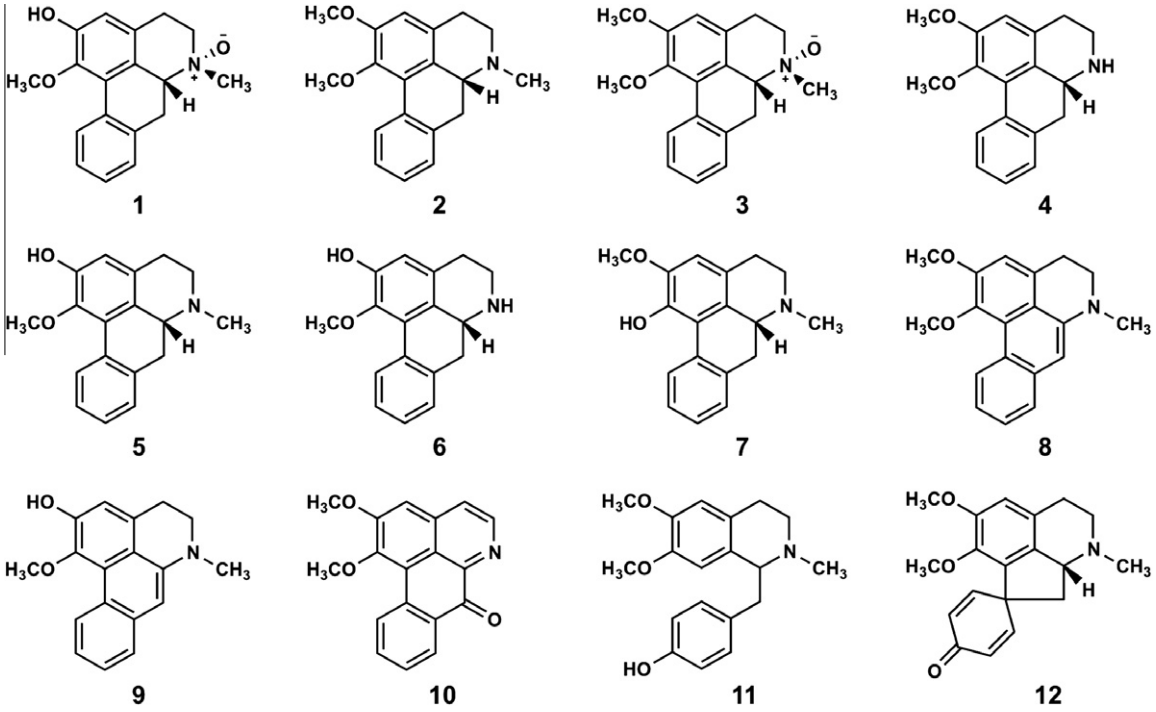

Figure 1. Alkaloids constituents from flower buds and leaves.

Table 3
Inhibitory effects of the MeOH extract (rt), Et_2O-, aq AcOH-, and alkaloid fractions from the flower buds of *N. nucifera* on melanogenesis in B16 melanoma 4A5 cells

Concn (μg/mL)	Inhibition (%)						IC_{50} (μg/mL)
	Control	1	3	10	30	100	
MeOH extract (rt)[a]	0.0 ± 2.3	−13.3 ± 1.7	−4.4 ± 2.6	5.2 ± 2.4	42.1 ± 2.8**	82.3 ± 0.3**	35.7
Et_2O fraction[b]	0.0 ± 2.4	1.7 ± 3.1	3.6 ± 1.3	5.2 ± 2.3	−0.3 ± 3.6	17.1 ± 3.8	—
aq AcOH fraction[a]	0.0 ± 3.2	−6.8 ± 3.1	7.0 ± 1.4	27.8 ± 1.5**	52.0 ± 0.9**	86.2 ± 0.5**	25.9
Alkaloids fraction[c]	0.0 ± 2.1	14.9 ± 1.9**	36.0 ± 1.2**	76.8 ± 0.5**	82.4 ± 0.3**	—	4.2

Each value represets the mean ± S.E.M., (n = 4).
Significantly different from the control group, *p < 0.05, **p < 0.01.
[a] The cell viabilities for the MeOH extract (rt) and its aq AcOH fraction at 30 and 100 μM are more than 86%.
[b] The cell viability for the the Et_2O fraction at 30 and 100 μM are 86.2% and 68.6%.
[c] The cell viability for the the alkaloids fraction at 10 and 30 μM are 94.4% and 55.2%.

(**19a**, **19b**, **23**) were examined. Compounds **19a**, **19b**, and **23** were synthesized according to previous reports (Fig. 3).[44-51] As shown in Table 5, a simple isoquinoline (**13**) and N-methylated isoquinoline (**14**) did not show inhibitory activity. Thus, the benzylisoquinoline or aporphine structure appears to be essential for inhibition. The synthesized benzylisoquinoline- and aporphine-type alkaloids (**19a**, **23**) showed potent inhibition with IC_{50} values of 5.9 and 5.0 μM, respectively, and a N-methylated aporphine-type alkaloid

Table 4
^{13}C NMR (150 MHz, CD_3OD) date for **1**

Position	1	Position	1
1	145.9	8	129.4
2	151.7	9	128.9
3	116.0	10	128.4
3a	128.2	11	128.7
4	25.1	11a	132.6
5	65.2	11b	128.3
6a	72.1	11c	121.9
7	30.7	N-CH_3	58.0
7a	135.3	O-CH_3	60.4

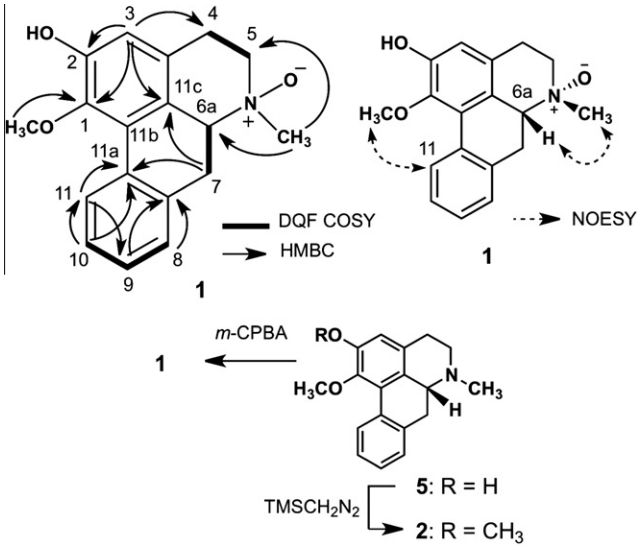

Figure 2. Structure of *N*-methylasimilobine *N*-oxide (**1**).

(**19b**) with a methoxy group at the 2-position showed more potent activity than **19a** and **23**. These findings suggest that the *N*-methyl group in the aporphine- or benzylisoquinoline-type skeleton is essential for greater inhibition of melanogenesis, and an oxygen group at the 2-position enhances this activity.

2.4. Effects of nuciferine (2) and *N*-methylasimilobine (5) on the expression of tyrosinase and TRP-1 and TRP-2 mRNA

Tyrosinase and tyrosinase-related protein 1 (TRP-1) and TRP-2 are known to catalyze the major steps in melanin synthesis.[52] Recently, we reported that several flavonoids inhibited the mRNA expression of tyrosinase and TRP-1 and -2.[3,4] In the present study, to clarify the mechanism of action of the principal alkaloid constituents **2** and **5** that had potent melanogenesis inhibitory activity, we examined their effects on the mRNA expression of tyrosinase and TRP-1 and -2 in B16 melanoma 4A5 cells. As a result, **2** and **5** were found to inhibit the expression of tyrosinase mRNA at 3–30 μM. In addition, **5** inhibited the expression of TRP-1 mRNA at 3–30 μM, whereas **2** inhibited the expression of TRP-2 mRNA at 10 and 30 μM (Table 6). However, **2** and **5** did not inhibit the enzyme activity of mushroom tyrosinase (less than 3% inhibition at 100 μM). Collectively, these findings suggest that the inhibition of melanogenesis by **2** and **5** is partly mediated by the reduced expression of tyrosinase and TRP-1 and TRP-2 mRNA, but the detailed mechanism of action remains to be studied further.

3. Experimental

3.1. General experimental procedures

The following instruments were used to obtain physical data: specific rotations, a Horiba SEPA-300 digital polarimeter (l = 5 cm); IR spectra, a Shimadzu FTIR-8100 spectrometer; FABMS

Table 5
Inhibitory effects of alkaloid constituents, **2**–**14**, **19a**, **19b**, and **23** from flower buds and leaves of *N. nucifera* on melanogenesis in B16 melanoma 4A5 cells

(μM)	Inhibition (%)						IC_{50} (μM)
	Control	1	3	10	30	100	
2[a,e]	0.0 ± 2.9	11.5 ± 0.2**	16.5 ± 1.7**	32.1 ± 1.5**	72.3 ± 2.8**	89.3 ± 0.5**	15.8
3[a,c]	0.0 ± 5.8	−4.8 ± 4.2	−3.2 ± 7.5	−0.1 ± 5.7	33.7 ± 2.5**	88.4 ± 0.8**	ca. 43
4[a,c]	0.0 ± 2.3	5.8 ± 1.7	16.2 ± 4.9**	12.1 ± 2.7*	17.6 ± 1.8**	91.9 ± 1.9**	62.9
5[a,c]	0.0 ± 1.9	15.4 ± 3.8**	19.7 ± 2.5**	37.9 ± 1.7**	70.6 ± 1.0**	90.5 ± 1.2**	14.5
6[a,e]	0.0 ± 2.1	7.4 ± 0.9	−7.4 ± 3.4	12.1 ± 4.5*	39.7 ± 1.8**	36.2 ± 1.8**	>100
7[a,f]	0.0 ± 2.8	−2.6 ± 2.0	11.1 ± 2.0**	27.3 ± 1.1**	65.9 ± 0.7**	—	19.3
8[b,d]	0.0 ± 7.2	14.5 ± 4.2	4.8 ± 2.3	11.8 ± 1.9	8.0 ± 5.1	78.4 ± 0.8**	ca. 85
9[a,e]	0.0 ± 1.9	10.9 ± 1.3	13.2 ± 3.3*	37.6 ± 2.7**	87.4 ± 5.0**	78.7 ± 0.5**	13.3
10[b,f]	0.0 ± 6.0	4.8 ± 3.7	8.7 ± 3.7	−1.5 ± 2.0	65.3 ± 0.9**	—	—
11[a,c]	0.0 ± 2.8	7.5 ± 1.3	18.2 ± 3.5**	34.0 ± 1.5**	50.4 ± 1.9**	80.3 ± 1.2**	25.6
12[a,c]	0.0 ± 2.4	4.0 ± 4.1	16.8 ± 2.7**	18.3 ± 2.6**	40.3 ± 0.9**	66.4 ± 0.6**	47.9
13[a,e]	0.0 ± 0.8	−12.6 ± 3.1	−41.5 ± 4.4	−54.2 ± 2.3	2.8 ± 2.6	26.1 ± 3.4**	—
14[a,c]	0.0 ± 4.2	10.6 ± 0.9	9.7 ± 3.5	9.4 ± 2.8	16.5 ± 2.0**	15.9 ± 0.8**	—
19a[a,f]	0.0 ± 2.8	30.0 ± 1.9**	42.7 ± 2.7**	59.2 ± 1.4**	72.2 ± 0.4**	22.3 ± 3.0**	5.9
19b[g]	0.0 ± 1.8	39.1 ± 2.7**	54.7 ± 5.9**	76.0 ± 2.0**	82.5 ± 2.1**	—	2.0
23[a,d]	0.0 ± 3.7	24.5 ± 1.0**	40.5 ± 3.4**	59.7 ± 1.6**	51.8 ± 1.1**	75.4 ± 0.8**	5.0
	Control	10	30	100	300	1000	
Arbutin[a]	0.0 ± 1.4	10.6 ± 0.6**	20.4 ± 0.5**	38.1 ± 0.9**	61.5 ± 0.6**	83.7 ± 0.5**	174

Each value represents the mean ± S.E.M., (n = 4).
Significantly different from the control group, *p < 0.05, **p<0.01.
[a] The cell viability at 30 μM is more than 86%.
[b] The cell viability at 30 μM is more than 69%.
[c] The cell viability at 100 μM is more than 82%.
[d] The cell viability at 100 μM is more than 77%.
[e] The cell viability at 100 μM is more than 46%.
[f] The cell viabilities for **7**, **10**, and **19a** at 100 μM are 39.7%, 22.5%, and 41.1%.
[g] The cell viabilities for **19b** at 10 and 30 μM are 98.0% and 76.7%.

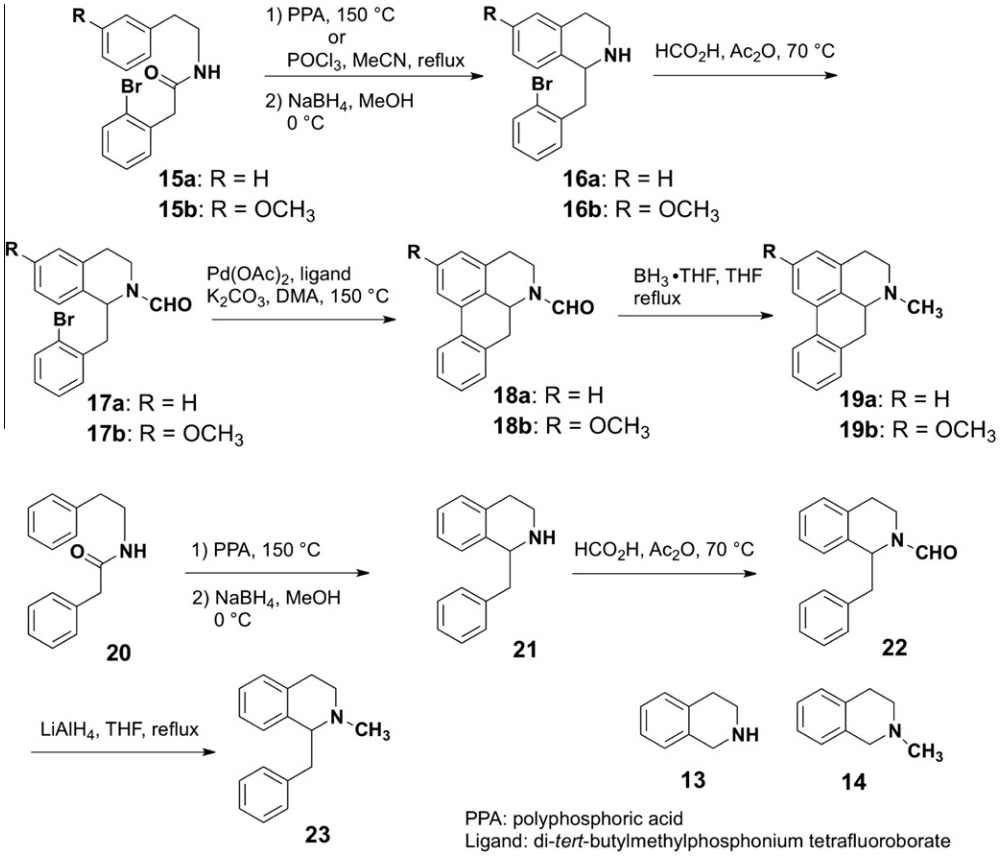

Figure 3. Synthesis of isoquinoline-type alkaloids.

Table 6
Effects of **2** and **5** on expression of tyrosinase and TRP-1 and 2 mRNA in B16 melanoma 4A5 cells

	Concentration (μM)			
	0	3	10	30
Tyrosinase mRNA/β-actin mRNA				
2	1.00 ± 0.05	0.61 ± 0.03**	0.64 ± 0.01**	0.38 ± 0.00**
5	1.00 ± 0.02	0.65 ± 0.04**	0.76 ± 0.04**	0.53 ± 0.01**
TRP-1 mRNA/β-actin mRNA				
2	1.00 ± 0.05	0.76 ± 0.08	0.74 ± 0.13	0.36 ± 0.02**
5	1.01 ± 0.02	0.79 ± 0.08**	0.72 ± 0.02**	0.49 ± 0.03**
TRP-2 mRNA/β-actin mRNA				
2	1.01 ± 0.00	1.11 ± 0.06	0.71 ± 0.07**	0.65 ± 0.07**
5	1.00 ± 0.02	1.10 ± 0.04	0.96 ± 0.05	0.88 ± 0.02*

Each value represents the mean ± S.E.M., (n = 4).
Significantly different from the control group, *p < 0.05, **p < 0.01.

and HRFABMS, a JEOL JMS-SX 102A mass spectrometer; ^{1}H NMR spectra, JEOL JNM LA 500 (500 MHz) and JEOL JNM ECA 600 (600 MHz) spectrometers; ^{13}C NMR spectra, JEOL JNM LA 500 (125 MHz) and JEOL JNM ECA 600 (150 MHz) spectrometers; and HPLC, a Shimadzu RID-6A refractive index and SPD-10A*vp* UV–vis detectors. A COSMOSIL 5C_{18}-MS-II and YMC-pack ODS-A {[250 × 4.6 mm id (5 μm) for analytical purposes] and [250 × 20 mm id (5 μm) for preparative purposes], Nacalai Tesque and YMC} column was used. The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd, 150–350 mesh); reverse-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary-phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reverse-phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

3.2. Plant material

The flower buds and leaves of *N. nucifera*, which was cultivated in Khon Kaen province, Thailand, were purchased from Mae Chu Co., Ltd (Nara, Japan) via Kwanjit Farm Co., Ltd (Nakhon Ratchasima, Thailand) at 2010. The stamens and seed of *N. nucifera* were collected by Dr. Y. Pongpiriyadacha (Rajamangala University of Technology) in Nakhonsi Thammarat province, Thailand at 2010. Voucher specimens (flower buds, leaves, stamens, and seeds) of this plant are on file in our laboratory.

3.3. Extraction and isolation

(1) The flower buds (1.0 kg), leaves (0.9 kg), stamens (1.0 g) and seeds (1.0 g) were powdered and extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided the MeOH extracts (155.0, 157.4, 0.13, 0.11 g, respectively). The MeOH extracts (145.0, 150.6 g) of the flower buds and leaves were partitioned into on EtOAc–H_2O (1:1, v/v) mixture to furnish the EtOAc-soluble fractions (31.2, 39.9 g) and aqueous fraction. The aqueous fractions were extracted with 1-BuOH to give the 1-BuOH- (41.0, 34.3 g) and H_2O- (72.8 g, 76.4 g) soluble fractions.

(2) The EtOAc-soluble fraction (31.2 g) from the flower buds was subjected to normal-phase silica gel column chromatography (1.0 kg, *n*-hexane→*n*-hexane-EtOAc→EtOAc→$CHCl_3$–MeOH–H_2O) followed by reversed-phase silica gel column chromatography (MeOH–H_2O) and finally HPLC (column: COSMOSIL $5C_{18}$-MS-II, eluent: MeOH–H_2O) to give nuciferine (**2**, 148 mg), *N*-nornuciferine (**4**, 11.2 mg), lysicamine (**10**, 36.5 mg), kaempferol 3-*O*-β-D-glucopyranoside (6.1 mg), isorhamnetin 3-*O*-β-D-glucopyranoside (81.0 mg), β-sitosterol (141 mg), and palmitic acid (96.3 mg). The 1-BuOH-soluble fraction (40.0 g) from the flower buds was subjected to normal-phase silica gel column chromatography (1.6 kg, $CHCl_3$–MeOH–H_2O) followed by preparative TLC (silica-gel 60F, $CHCl_3$–MeOH–H_2O = 8:3:1) and HPLC (COSMOSIL $5C_{18}$-MS-II, MeOH–H_2O in 1% AcOH) to give *N*-methylasimilobine (**5**, 6.6 mg), lysicamine (**10**, 102 mg), and pronuciferine (**12**, 56.0 mg).

(3) The EtOAc-soluble fraction (36.9 g) from the leaves was subjected to normal-phase silica gel column chromatography (1.6 kg, $CHCl_3$–MeOH→$CHCl_3$–MeOH–H_2O) followed by reversed-phase silica gel column chromatography (MeOH–H_2O) and finally HPLC (YMC-pack ODS-A, MeOH–H_2O in 1% AcOH) to give *N*-methylasimilobine *N*-oxide (**1**, 3.3 mg), nuciferine (**2**, 67.3 mg) nuciferine *N*-oxide (**3**, 40.7 mg), *N*-nornuciferine (**4**, 2.3 mg), dehydronuciferine (**8**, 3.9 mg), lysicamine (**10**, 41.8 mg), quercetin 3-*O*-β-D-glucopyranoside (10.2 mg), quercetin 3-*O*-β-D-galactopyranoside (7.5 mg), and (+)-catechine (40.5 mg). The 1-BuOH-soluble fraction (31.3 g) was subjected to normal-phase silica gel column chromatography (1.3 kg, $CHCl_3$–MeOH→$CHCl_3$–MeOH–H_2O) followed by reversed-phase silica gel column chromatography (MeOH–H_2O) and finally HPLC (YMC-pack ODS-A, MeOH–H_2O in 1% AcOH) to give nuciferine (**2**, 83.0 mg), nuciferine *N*-oxide (**3**, 22.1 mg), *N*-methylasimilobine (**5**, 282 mg), asimilobine (**6**. 149 mg), (−)-lirinidine (**7**, 7.2 mg), 2-hydroxy-1-methoxy-6a,7-dehydroaporphine (**9**, 2.9 mg), lysicamine (**10**, 3.0 mg), D,L-armepavine (**11**, 27.4 mg), and pronuciferine (**12**, 8.3 mg).

(4) The flower buds (0.9 kg) were powdered and extracted four times with MeOH at room temperature for 7 h followed by evaporation under reduced pressure to produce the MeOH extract (60.8 g). The MeOH extract (60.0 g) was partitioned with Et_2O-1% aqueous AcOH mixture and the 1% aqueous AcOH fraction was made basic to litmus with 10% NH_4OH solution. The alkaline solution was extracted with Et_2O and the Et_2O fraction was washed with 1% aqueous NaOH to furnish the Et_2O-soluble fraction (crude alkaloid fraction, 4.21 g). The crude alkaloid fraction (3.52 g) from the flower buds was purified by ordinary and reversed-phase silica gel column chromatographies followed by HPLC (COSMOSIL $5C_{18}$-MS-II, MeOH–H_2O in 1% AcOH) to provide nuciferine (**2**, 183 mg), nornuciferine (**4**, 121 mg), *N*-methylasimilobine (**5**, 36.0 mg), (−)-lirinidine (**7**, 3.0 mg), lysicamine (**10**, 38.2 mg), pronuciferine (**12**, 23.0 mg), and β-sitosterol (1.8 mg). The known compounds were identified by comparison of their physical data ($[\alpha]_D$, ^1H NMR, ^{13}C NMR, and MS) with reported values.

3.4. *N*-Methylasimilobine *N*-oxide

A pale yellow oil; $[\alpha]_D^{24}$ −56.3° (*c* 0.16, MeOH); IR (film): ν_{max} 3400, 1509 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ 2.76, 3.50 (2H, m, 4-H_2), 3.20 (1H, dd, *J* = 4.0, 13.7 Hz, 7a-H), 3.28 (1H, m, 7b-H), 3.39 (3H, s, O←N-CH_3), 3.56 (3H, s, OCH_3), 3.56, 3.73 (2H, m, 5-H_2), 4.44 (1H, dd, *J* = 4.0, 14.0 Hz, 6a-H), 6.71 (1H, s, 3-H), 7.25 (1H, dd like, *J* = 7.6, 7.6 Hz, 9-H), 7.30 (1H, dd like, *J* = 7.6, 7.6 Hz, 10-H), 7.37 (1H, d like, *J* = 7.6 Hz, 8-H), 8.31 (1H, d like, *J* = 7.6 Hz, 11-H); ^{13}C NMR: given in Table 4; EIMS *m*/*z* 297 [M^+]; HREIMS *m*/*z* 297.1369 (Calcd for $C_{18}H_{19}NO_3$ [M^+], *m*/*z* 297.1365).

3.5. Oxidation of *N*-methylasimilobine (5)

Solution of *N*-methylasimilobine (**5**, 4.0 mg, 0.014 mmol) in $CHCl_3$ (1.0 mL) was treated with *m*-CPBA (75%, wet with H_2O, 9.8 mg, 0.043 mmol), and the mixture was stirred at room temperature for 2 h. Removal of the solvent from the reaction mixture under reduced pressure furnished a residue, which was purified by normal-phase silica gel column chromatography [$CHCl_3$–MeOH–H_2O = 7:3:1, lower phase] and HPLC [YMC-pack ODS-A, MeOH–H_2O in 1% AcOH (50:50)] to give *N*-methylasimilobine *N*-oxide (**1**, 1.9 mg). *N*-methylasimilobine *N*-oxide was identified by comparison of the physical data ($[\alpha]_D$, ^1H NMR, ^{13}C NMR, and MS) with that of natural form of *N*-methylasimilobine *N*-oxide.

3.6. Methylation of *N*-methylasimilobine (5)

Solution of *N*-methylasimilobine (**5**, 5.0 mg, 0.018 mmol) in MeCN (0.5 mL) and MeOH (0.5 mL) was treated with *N*,*N*-diisopylethylamine (0.006 mL, 0.036 mmol) and trimethylsilyldiazomethane (2 M in hexane, 0.018 mL, 0.036 mmol), and the mixture was stirred at room temperature for 15 h. Removal of the solvent from the reaction mixture under reduced pressure furnished a residue, which was purified by normal-phase silica gel column chromatography [$CHCl_3$–MeOH–H_2O = 7:3:1, lower phase] to give nuciferin (**2**, 4.0 mg). Nuciferin was identified by comparison of the physical data (($[\alpha]_D$, ^1H NMR, ^{13}C NMR, and MS) with that of natural form of nuciferin.

3.7. 1-[(2-Bromophenyl)methyl]-3,4-dihydro-1*H*-isoquinoline-2-carboxaldehyde (17a, R = H)

According to the protocol reported,[25,44] a mixture of 2-(bromophenyl)-*N*-(2-phenylethyl)acetamide (**15a**, 2.9 g, 9.1 mmol) and polyphosphoric acid (19 g) was heated at 150 °C for 12 h. After being cooled, the reaction mixture was diluted with H_2O (50 mL), and the resulting mixture was neutralized with aqueous $NaHCO_3$ and extracted with Et_2O. The extract was washed with brine and condensed to give a pale brown oil (2.8 g), to which was added $NaBH_4$ (830 mg, 22 mmol) in MeOH (10 mL), and the mixture was stirred at 0 °C for 3 h. The reaction mixture was poured into brine (50 mL) and extracted with CH_2Cl_2. The extract was washed with brine and condensed to give 1-[(2-bromophenyl)methyl]-1,2,3,4-tetrahydroisoquinoline (**16a**, 2.6 g) as a pale yellow oil. To the oil was added pre-prepared acetic formic anhydride obtained by heating a mixture of Ac_2O (8.1 mL) and formic acid (6.4 mL), and the mixture was stirred at 70 °C for 3 h. After being cooled, the reaction mixture was concentrated under reduced pressure. The residue was diluted with H_2O (20 mL) and the resulting mixture was extracted with $CHCl_3$. The extract was washed successively with aqueous $NaHCO_3$ and brine, and condensed to give a pale yellow oil (3.0 g), which on normal-phase silica gel column chromatography ($CHCl_3$) gave title compound **17a** (2.4 g, 80% from **15a**).

Viscous oil; IR (neat): ν_{max} 1668, 1431, 1292, 1196, 1157, 1026 cm^{-1}; ^1H NMR spectral analysis revealed the products to be a ca. 3.5:1 mixture of two amide rotamers. ^1H NMR (500 MHz, $CDCl_3$) δ 2.83–2.90 (0.22 H, m, 4a-H), 2.87 (0.78 H, ddd-like, *J* = ca. 16.3, 4.6, 2.6, 4a-H), 2.92–2.98 (0.22 H, m, 4b-H), 2.98 (0.78H, ddd, *J* = 16.3, 11.5, 6.3, 4b-H), 3.11 (0.78H, dd, *J* = 14.0, 10.9, benzylic methylene), 3.17 (0.22H, dd, *J* = 14.0, 9.4, benzylic methylene), 3.27 (0.78H, ddd, *J* = 13.2, 11.5, 4.6, 3a-H), 3.37 (0.78H, dd, *J* = 14.0, 3.5, benzylic methylene), 3.39 (0.22H, dd, *J* = 14.0, 4.9, benzylic methylene), 3.68–3.72 (0.44H, m, 3a-H and 3b-H), 4.52 (0.78H, ddd,

J = 13.2, 6.3, 2.6, 3b-H), 4.89 (0.78H, dd, J = 10.9, 3.5, H-1), 5.79 (0.22H, dd, J = 9.4, 4.9, H-1), 7.03–7.63 (8H, m, arom.), 7.50 (0.78H, s, CHO), 8.02 (0.22H, s, CHO); ^{13}C NMR (125 MHz, $CDCl_3$, major rotamer/minor rotamor) δ 28.1/29.7 (4-C), 34.3/40.2 (3-C), 43.4/41.9 (benzylic methylene), 56.8/51.1 (1-C), 126.6/126.9/127.0/127.2/127.4/127.5/127.9/128.4/129.0/129.1/129.3/131.4132.1/132.8/133.2 (d, arom.), 124.4/125.3/133.0/133.9/135.5/135.6/136.6/137.0 (s, arom.), 161.3 (*C*HO).

3.8. 5,6,6a,7-Tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline-6-carboxaldehyde (18a, R = H)

According to the protocol reported,[25] a mixture of compound **17a** (1.0 g, 3.0 mmol), K_2CO_3 (836 mg, 6.1 mmol), di-*tert*-butylmethylphosphonium tetrafluoroborate (226 mg, 0.9 mmol), and $Pd(OAc)_2$ (137 mg, 0.6 mmol) in DMA (5 mL) was heated at 150 °C for 12 h. The reaction mixture was concentrated under reduced pressure, and the residue was diluted with $CHCl_3$. The $CHCl_3$-insoluble material was filtered off, and the filtrate was condensed to give a brown oil (893 mg), which on normal-phase silica gel column chromatography ($CHCl_3$) gave title compound **18a** (350 mg, 46%).

Pale brown solid; mp 133–135 °C; IR (neat): ν_{max} 1662, 1427, 1392, 1256, 1242, 1184, 1123, 1041 cm^{-1}; ^{1}H NMR analysis revealed the products to be a ca. 2:1 mixture of two amide rotamers. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.83 (0.66H, dd, H = 14.3, 14.1, 7a-H), 2.86 (0.66H, br d-like, J = ca. 15.8, 4a-H), 2.81–2.91 (1.02H, m, 4a-H, 4b-H and 7a-H), 2.96 (0.66H, ddd-like, J = ca. 15.8, 12.6, 4.6, 4b-H), 3.16 (0.34H, J = 12.6, 10.3, 4.0, 5a-H), 3.19 (0.34H, dd, J = 14.6, 14.6, 7b-H), 3.27 (0.66H, dd, J = 14.1, 4.6, 7b-H), 3.43 (0.66H, ddd, J = 12.6, 12.6, 2.9, 5a-H), 3.87 (0.66H, ddd, J = 12.6, 4.6, 2.0, 5b-H), 4.50 (0.34H, ddd, J = 12.6, 4.3, 3.5, 5b-H), 4.70 (0.34H, dd, J = 14.6, 4.6, 6a-H), 5.13 (0.66H, dd, J = 14.3, 4.6, 6a-H), 7.08–7.81 (6H, m, arom.), 8.29 (0.66H, s, CHO), 8.42 (0.34H, s, CHO); ^{13}C NMR (125 MHz, $CDCl_3$, major rotamer/minor rotamor) δ 31.0/29.6 (4-C), 33.0/37.1 (7-C), 42.1/36.1 (5-C), 49.3/53.1 (6a-C), 122.7/122.8/123.7/124.0/127.3/127.5/127.6/127.7/127.9/128.1/128.2/128.6/129.1 (d, arom.), 130.9/131.4/133.5/133.7/133.8/134.2/134.3/134.5/134.6/134.9 (s, arom.), 162.2/162.0 (*C*HO); positive-ion FAB-MS: m/z 250 [M+H]$^+$.

3.9. 6-Methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline (19a, R = H)

A mixture of compound **18a** (70 mg, 0.28 mmol) and ca. 0.9 M solution of THF-boran complex (1 mL, 0.9 mmol) in THF (4 mL) was heated under reflux for 6 h. The reaction was quenched with 2 N HCl (1 mL), and the mixture was made alkaline by addition of 1 N aqueous NaOH (30 mL). The resulting mixture was then heated under reflux for 4 h, and extracted with EtOAc. The extract was washed with brine and condensed to give a pale brown oil, which on normal-phase silica gel column chromatography ($CHCl_3$) gave title compound **19a** (47 mg, 71%).

Pale yellow oil; The ^{1}H NMR spectroscopic properties of **19a** were in accord with those reported,[46] and signals were unambiguously assigned in the present study. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.54 (1H, ddd-like, J = ca. 11.5, 11.5, 3.8, 5a-H), 2.56 (3H, s, NCH_3), 2.70 (1H, dd, J = 14.0, 14.0, 7a-H), 2.76 (1H, br dd, J = ca. 15.0, 3.8, 4a-H), 3.07 (1H, ddd, J = 11.5, 5.8, 1.5, 5b-H), 3.17 (1H, dd, J = 14.0, 4.6, 7b-H), 3.17–3.23 (1H, m, 4b-H), 3.23 (1H, dd, J = 14.0, 4.6, 6a-H), 7.07 (1H, d-like, J = 7.5, arom.), 7.21–7.28 (3H, m, arom.), 7.31 (1H, ddm, J = ca. 7.8, 7.0, arom.), 7.76 (1H, d, J = 7.8, arom.), 7.71 (1H, d, J = 7.6, arom.); ^{13}C NMR (125 MHz, $CDCl_3$) δ 29.1 (4-C), 34.1 (7-C), 44.0 (NCH_3), 53.4 (5-C), 62.0 (6a-C), 121.8/123.7/126.8/127.3/127.5/128.0/128.4 (d, arom.), 133.4/133.5/133.8/134.3/135.3 (s, arom.).

3.10. 1-[(2-Bromophenyl)methyl]-3,4-dihydro-6-methoxy-1*H*-isoquinoline-2-carboxaldehyde (17b, R = OCH_3)

According to the protocol reported,[25] a mixture of 2-(bromophenyl)-*N*-[2-(2-methoxyphenyl)ethyl]acetamide (**15b**, 500 mg, 1.43 mmol) and phosphorous oxychloride (0.5 mL, 0.53 mmol) in MeCN (7 mL) was heated under reflux for 12 h. After being cooled, the reaction mixture was neutralized with aqueous $NaHCO_3$, and extracted with Et_2O. The extract was washed with brine and condensed to give a pale brown oil (537 mg), to which was added with $NaBH_4$ (82 mg, 2.16 mmol) in MeOH (7 mL), and the mixture was stirred at 0 °C for 3 h. The reaction mixture was poured into brine (20 mL) and extracted with CH_2Cl_2. The extract was washed with brine, and condensed to give 1-[(2-bromophenyl)methyl]-6-methoxy-1,2,3,4-tetrahydroisoquinoline (**16b**, 478 mg) as a pale yellow oil. To the oil was added pre-prepared acetic formic anhydride obtained by heating a mixture of Ac_2O (1.3 mL) and formic acid (1.0 mL), and the mixture was stirred at 70 °C for 1 h. After being cooled, the reaction mixture was concentrated under reduced pressure. The residue was diluted with H_2O (20 mL) and the resulting mixture was extracted with $CHCl_3$. The extract was washed successively with aqueous $NaHCO_3$ and brine, and condensed to give a pale yellow oil (438 mg), which on normal-phase silica gel column chromatography (n-hexane–EtOAc = 10:1→5:1) gave title compound **17b** (385 mg, 96%).

Viscous oil; IR (neat): ν_{max} 1670, 1612, 1504, 1431, 1284, 1257, 1234, 1157, 1022 cm^{-1}; ^{1}H NMR analysis revealed the products to be a ca. 4:1 mixture of two amide rotamers. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.79–2.84 (0.2 H, m, 4a-H), 2.83 (0.8 H, ddd-like, J = ca. 16.3, 5.1, 2.6, 4a-H), 2.88–2.95 (0.2 H, m, 4b-H), 2.95 (0.8H, ddd, J = 16.3, 11.5, 6.3, 4b-H), 3.07 (0.8H, dd, J = 14.0, 10.6, benzylic methylene), 3.13 (0.2H, dd, J = 14.0, 9.5, benzylic methylene), 3.26 (0.8H, ddd, J = 13.2, 11.5, 5.1, 3a-H), 3.33 (0.8H, dd, J = 14.1, 3.5, benzylic methylene), 3.34 (0.2H, dd, J = 14.0, 4.9, benzylic methylene), 3.64–3.69 (0.4H, m, 3a-H and 3b-H), 3.79 (0.6H, s, OCH_3), 3.81 (2.4H, s, OCH_3), 4.50 (0.8H, ddd, J = 13.2, 6.3, 2.6, 3b-H), 4.83 (0.8H, dd, J = 10.6, 3.5, 1-H), 5.73 (0.22H, dd, J = 9.5, 4.9, 1-H), 6.72–7.63 (7H, m, arom.), 7.49 (0.8H, s, C*H*O), 8.01 (0.2H, s, C*H*O); ^{13}C NMR (125 MHz, $CDCl_3$, major rotamer/minor rotamor) δ: 28.5/30.0 (4-C), 34.1/40.2 (3-C), 43.5/42.0 (benzylic methylene), 55.3/55.2 (OCH_3), 56.4/50.1 (1-C), 112.9/113.0/113.4/113.5/127.2/127.8/128.0/128.4/128.5/129.0/131.5/132.1/132.7/133.1 (d, arom.), 124.4/125.3/127.7/127.8/134.4/135.3/136.7/137.1/158.3/158.6/ (s, arom.), 161.31/168.28 (*C*HO).

3.11. 2-Methoxy-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline-6-carboxaldehyde (18b, R = OCH_3)

In a similar manner used for the preparation of **18a**, compound **18b** (370 mg, 1.0 mmol) was subjected to coupling reaction to give a pale yellow oil (356 mg), which on normal-phase silica gel column chromatography (n-hexane-Et_2O, 2:1) gave title compound **18b** (131 mg, 46%).

Pale yellow solid; mp 171–173 °C; IR (KBr): ν_{max} 1662, 1608, 1427, 1400, 1319, 1246, 1195, 1161, 1049 cm^{-1}; ^{1}H NMR spectral analysis revealed the products to be a ca. 2:1 mixture of two amide rotamers; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.79 (0.66H, dd, H = 14.0, 14.0, 7a-H), 2.79–2.85 (0.66H, m, 4a-H), 2.85–2.87 (0.68H, m, 4a-H and 4b-H), 2.89 (0.34H, dd, J = 14.3, 4.6, 7a-H), 2.95 (0.66H, ddd-like, J = ca. 16.0, 12.3, 4.6, 4b-H), 3.14 (0.34H, dd, J = 14.6, 14.3, 7b-H), 3.18 (0.34H, J = 12.9, 10.6, 4.0, 5a-H), 3.27 (0.66H, dd, J = 14.0, 4.6, 7b-H), 3.43 (0.66H, ddd, J = 12.3, 12.3, 2.6, 5a-H), 3.85 (0.66H, ddd, J = 12.3, 4.6, 1.8, 5b-H), 3.86 (3H, s, OCH_3), 4.47 (0.34H, ddd, J = 12.9, 4.6, 2.9, 5b-H), 4.66 (0.34H, dd, J = 14.6, 4.6, 6a-H), 5.07 (0.66H, dd, J = 14.0, 4.6, 6a-H), 6.63–7.77 (6H, m, arom.), 8.29 (0.66H, s, C*H*O), 8.42 (0.33H, s, C*H*O); ^{13}C NMR

(125 MHz, $CDCl_3$, major rotamer/minor rotamor) δ: 31.3/30.0 (4-C), 33.3/37.4 (7-C), 42.2/36.2 (5-C), 49.1/52.8 (6a-C), 109.0/109.1/112.6/112.8/123.7/124.0/127.4/127.9/128.2/128.3/128.7/129.2 (d, arom.), 123.4/123.8/133.5/133.7/134.5/135.1/135.2/135.5/135.90/135.94/158.8/159.2 (s, arom.), 162.3/162.0 (*C*HO).

3.12. 6-Methyl-2-methoxy-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline (19b, R = OCH_3)

In a similar manner used for the preparation of **19a**, compound **18b** (70 mg, 0.25 mmol) was reduced with THF-boran complex to give a pale yellow oil (72 mg), which on normal-phase silica gel column chromatography (*n*-hexane–acetone = 2:1→1:1) gave title compound **19b**[46,47] (46 mg, 69%).

Pale yellow oil; IR (neat): ν_{max} 1608, 1454, 1453, 1357, 1319, 1246, 1123, 1072 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 2.54 (1H, ddd-like, *J* = ca. 11.7, 11.7, 4.0, 5a-H), 2.56 (3H, s, NC*H*$_3$), 2.69 (1H, dd, *J* = 13.5, 13.5, 7a-H), 2.73 (1H, br dd, *J* = ca. 15.0, 4.0 4a-H), 3.08 (1H, ddd, *J* = 11.7, 6.1, 1.5, 5b-H), 3.16 (1H, dd, *J* = 13.5 4.6, 7b-H), 3.19 (1H, dd, *J* = 13.5, 4.6, 6a-H), 3.21 (1H, ddd-like, *J* = ca. 15.0, 11.7, 6.1, 4b-H), 3.85 (3H, s, OC*H*$_3$), 6.63 (1H, d, *J* = 2.6, arom.), 7.12 (1H, d, *J* = 2.6, arom.), 7.22–7.34 (3H, m, arom.), 7.69 (1H, dd, *J* = ca. 7.5, arom.); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 29.7 (4-C), 34.4 (7-C), 43.7 (N*C*H$_3$), 53.4 (5-C), 55.3 (O*C*H$_3$), 61.7 (6a-C), 108.2/112.5/123.7/127.3/127.7/128.5 (d, arom.), 126.4/134.1/134.8/134.9/135.6/158.6 (s, arom.). Positive-ion FABMS: *m*/*z* 266 $[M+H]^+$, FABHRMS *m*/*z* 266.1548 (Calcd for $C_{18}H_{20}NO$ $[M+H]^+$, *m*/*z* 266.1545).

3.13. 1-Phenylmethy-3,4-dihydro-1*H*-isoquinoline-2-carboxaldehyde (22)

In a similar manner used for the preparation of **16a**, the Bischler–Napieralski reaction of *N*-(2-phenylethyl)benzeneacetamide (**20**,[48] 460 mg, 1.93 mmol) by using polyphosphoric acid, and subsequent $NaBH_4$ reduction of the resulting dihydroispquinoline derivative gave 1-phenylmethyl-1,2,3,4-tetrahydroisoquinoline (**21**),[49] to which was then acylated with acetic formic anhydride to give title compound **22** (490 mg), which was used for the next reaction without further purification. The ^1H NMR spectral properties were in accord with those reported.[50,51]

3.14. 2-Methyl-1-phenylmethyl-1,2,3,4-tetrahydroisoquinoline (23)

According to the literature,[51] a mixture of the crude compound **22** (100 mg) and $LiAlH_4$ (30 mg, 0.8 mmmol) in THF (1 mL) was heated under reflux for 1 h. Work-up gave a pale brown oil (84 mg), which on normal-phase silica gel column chromatography ($CHCl_3$–MeOH = 100:1) gave title compound **23**[51] (74 mg, 79% from **20**).

Pale yellow oil; ^1H NMR (500 MHz, $CDCl_3$) δ 2.66 (1H, ddd, *J* = 16.3, 4.9, 4.9, 4a-H), 2.76 (1H, ddd, *J* = 12.6, 5.2, 4.9, 3a-H), 2.88 (1H, ddd, *J* = 16.3, 8.6, 5.2, 4b-H), 2.89 (1H, dd, *J* = 13.8, 6.3, benzylic methlene), 3.15 (1H, *J* = 13.7, 5.7, benzylic methylene), 3.20 (1H, *J* = 12.6, 8.6, 4.9, 3b-H), 3.81 (1H, dd, *J* = 6.3, 5.7, 6a-H), 6.75 (1H, d, *J* = 7.4, arom.), 7.00–7.26 (8H, m, arom.); ^{13}C NMR (125 MHz, $CDCl_3$,) δ 25.9 (4-C), 41.5 (benzylic methylene), 42.8 (N*C*H$_3$), 47.0 (3-C), 65.1 (1-C), 125.3/125.9/126.0/127.9/128.0/128.7/129.6 (d, arom.), 134.3/137.8/140.0 (s, arom.).

3.15. Melanogenesis

Screening test for melanogensis using B16 melanoma 4A5 cells was performed as described previously[3] with slight modifications. The melanoma cells ($2.0\rightarrow10^4$ cells/400 μL/well) were seeded into 24-well multiplates. After 24 h of culture, a test compound and theophylline 1 mM were added and incubated for 72 h. The cells were harvested by incubating with PBS containing EDTA 1 mM and 0.25% trypsin, and then the cells were washed with PBS. The cells were treated with NaOH 1 M (120 μL/tube, 80 °C, 30 min) to yield a lysate, an aliquot (100 μL) of the lysate was transferred to a 96-well microplate, and the optical density of each well was measured with a microplate reader (Model 550, Bio-Rad Laboratories) at 405 nm (reference: 655 nm). The test compound was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. The production of melanin was corrected based on cell viability. Inhibition (%) was calculated using the following formula, and IC_{50} values were determined graphically.

$$\text{Inhibition}(\%) = [(A - B)/A]/(C/100) \times 100$$

where A and B indicate the optical density of vehicle- and test compound-treated groups, respectively, and C indicates cell viability (%).

3.16. Cell viability

The cell viability was assessed by our previous report[3] with a slight modification. The melanoma cells ($5.0\rightarrow10^3$ cells/100 μL/well) were seeded into 96-well microplates and incubated for 24 h. After 70-h incubation with theophylline 1 mM and a test compound, 10 μL of WST-8 solution (Cell Counting Kit-8™) was added to each well. After a further 2 h in culture, the optical density of the H_2O-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad Laboratories) at 450 nm (reference: 655 nm). The test compound was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the medium was 0.1%. Cell viability (%) and inhibition (%) were calculated using the following formula, and IC_{50} values were determined graphically.

$$\text{Cell viability}(\%) = A/B \times 100$$

where A and B indicate the optical density of vehicle- and test compound-treated groups, respectively.

3.17. Expression of tyrosinase, TRP-1, and TRP-2 mRNA

The melanoma cells (1.0×10^5 cells/2 mL/well) were seeded into 6-well multiplates and cultured for 24 h. The cells were then incubated with the test compound and theophylline 1 mM for 72 h. Total RNA was extracted from the cells using an RNeasy™ mini Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and determining the ratio of the readings at 260 and 280 nm. cDNAs were synthesized from 1 μg total RNA using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Template cDNA thus obtained was incubated with gene-specific primers and with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) in a Mini Opticon (Bio-Rad Laboratories). The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized to →-actin mRNA. The thermal cycling program had initial denaturation (95 °C for 2 min) and then 40 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). The primer pairs were: tyrosinase primers, 5′-CAGATCTCTGATGGCCAT-3′ and 5′-GGATGACATAGACTGAGC-3′; TRP-1, 5′-CTTTCTCCCTTCCTTACTGG-3′; 5′-TGGCTTCATTCTTGGTGCTT-3′; TRP-2, 5′-TGAGAAGAAACAAAGTAGGCAGAA-3′ and 5′-CAACCCCAAGAGCAAGACGAAAGC-3′; and β-actin primers, 5′-ATGGGTCAGAAGGACTCCTACG-3′ and 5′-AGTGGTACGACCAGAGGCATAC-3′.

3.18. Mushroom tyrosinase

The tyrosinase activity was determined as described previously[3] with a slight modification. Briefly, the incubation mixture contained 70 μL of L-DOPA (2.5 mM in the phosphate buffer) as a substrate, and 20 μL of the test compound solution was prepared. The reaction was initiated by the addition of 120 μL of tyrosinase (80.5 units/mL) at 25 °C for 5 min, and then the absorbance at 405 nm (reference: 655 nm) was measured with a microplate reader (model 550, Bio-Rad Laboratories). The amount of dopaquinone was determined based on the absorbance. The test compound was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. The following equation was used to calculate the inhibition of mushroom tyrosinase.

$$\text{Inhibition}(\%) = (A - B)/A \times 100$$

where A and B indicate the absorbance of vehicle- and test compound-treated groups, respectively.

3.19. Statistical analyses

Values are expressed as mean ± S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analyses

Acknowledgment

This research was supported in part by a Ministry of Education, Culture, Sports, Science and Technology (MEXT)-Supported Program for the Strategic Research Foundation at Private Universities.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.038.

References and notes

1. Nakamura, S.; Fujimoto, K.; Nakashima, S.; Matsumoto, T.; Miura, T.; Uno, K.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2012**, *60*, 752.
2. Mukherjee, P. K.; Mukherjee, D.; Maji, A. K.; Rai, S.; Heinrich, M. *J. Pharm. Pharmacol.* **2009**, *61*, 407.
3. Matsuda, H.; Nakashima, S.; Oda, Y.; Nakamura, S.; Yoshikawa, M. *Bioorg. Med. Chem.* **2009**, *17*, 6048.
4. Nakashima, S.; Matsuda, H.; Oda, Y.; Nakamura, S.; Xu, F.; Yoshikawa, M. *Bioorg. Med. Chem.* **2010**, *18*, 2337.
5. Nakamura, S.; Chen, G.; Nakashima, S.; Matsuda, H.; Pei, Y.; Yoshikawa, M. *Chem. Pharm. Bull.* **2010**, *58*, 690.
6. Fujimoto, K.; Nakamura, S.; Nakashima, S.; Matsumoto, T.; Uno, K.; Ohta, T.; Miura, T.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2012**, *60*, 1188.
7. Nakamura, S.; Moriura, T.; Park, S.; Fujimoto, K.; Matsumoto, T.; Ohta, T.; Matsuda, H.; Yoshikawa, M. *J. Nat. Prod.* **2012**, *75*, 1425.
8. Morikawa, T.; Li, X.; Nishida, E.; Nakamura, S.; Ninomiya, K.; Matsuda, H.; Hamao, M.; Muraoka, O.; Hayakawa, T.; Yoshikawa, M. *Chem. Pharm. Bull.* **2011**, *59*, 889.
9. Matsuda, H.; Nakamura, S.; Fujimoto, K.; Moriuchi, R.; Kimura, Y.; Ikoma, N.; Hata, Y.; Muraoka, O.; Yoshikawa, M. *Chem. Pharm. Bull.* **2010**, *58*, 1617.
10. Morikawa, T.; Wang, L. B.; Ninomiya, K.; Nakamura, S.; Matsuda, H.; Muraoka, O.; Wu, L. J.; Yoshikawa, M. *Chem. Pharm. Bull.* **2009**, *57*, 853.
11. Morikawa, T.; Wang, L. B.; Nakamura, S.; Ninomiya, K.; Yokoyama, E.; Matsuda, H.; Muraoka, O.; Wu, L. J.; Yoshikawa, M. *Chem. Pharm. Bull.* **2009**, *57*, 361.
12. Nakamura, S.; Okazaki, Y.; Ninomiya, K.; Morikawa, T.; Matsuda, H.; Yoshikawa, M. *Chem. Pharm. Bull.* **2008**, *56*, 1704.
13. Xie, Y.; Morikawa, T.; Ninomiya, K.; Imura, K.; Muraoka, O.; Yuan, D.; Yoshikawa, M. *Chem. Pharm. Bull.* **2008**, *56*, 1628.
14. Yoshikawa, M.; Li, X.; Nishida, E.; Nakamura, S.; Matsuda, H.; Muraoka, O.; Morikawa, T. *Chem. Pharm. Bull.* **2008**, *56*, 559.
15. Yoshikawa, M.; Morikawa, T.; Asao, Y.; Fujiwara, E.; Nakamura, S.; Matsuda, H. *Chem. Pharm. Bull.* **2007**, *55*, 606.
16. Yoshikawa, M.; Nakamura, S.; Kato, Y.; Matsuhira, K.; Matsuda, H. *Chem. Pharm. Bull.* **2007**, *55*, 598.
17. Yoshikawa, M.; Xu, F.; Morikawa, T.; Pongpiriyadacha, Y.; Nakamura, S.; Asao, Y.; Kumahara, A.; Matsuda, H. *Chem. Pharm. Bull.* **2007**, *55*, 308.
18. Morikawa, T.; Muraoka, O.; Yoshikawa, M. *Yakugaku Zasshi* **2010**, *130*, 673.
19. Hearing, V. J.; Körner, A. M.; Pawelek, J. M. *J. Invest. Dermatol.* **1982**, *79*, 16.
20. Hearing, V. J.; Jiménez, M. *Int. J. Biochem.* **1987**, *19*, 1141.
21. Prota, G. *Med. Res. Rev.* **1988**, *8*, 525.
22. Kuzumaki, T.; Matsuda, A.; Wakamatsu, K.; Ito, S.; Ishikawa, K. *Exp. Cell Res.* **1993**, *207*, 33.
23. Tomita, M.; Watanabe, Y.; Tomita, M.; Furukawa, H. *Yakugaku Zasshi* **1961**, *81*, 469.
24. Jackman, L. M.; Trewella, J. C.; Moniot, J. L.; Shamma, M.; Stephens, R. L.; Wenkert, E.; Leboeuf, M.; Cave, A. *J. Nat. Prod.* **1979**, *42*, 437.
25. Lafrance, M.; Blaquière, N.; Fagnou, K. *Eur. J. Org. Chem.* **2007**, 811.
26. Lu, S. T.; Wu, Y. C.; Leou, S. P. *J. Chin. Chem. Soc.* **1987**, *34*, 33.
27. The absolute configuration of the methyl group of three was not decided. Therefore, the configuration was clarified by NOESY experiment and the structure was shown in Figure 1.
28. Zheng, Z.; Wang, M.; Wang, D.; Duan, W.; Wang, X.; Zheng, C. *J. Chromatogr., B* **2010**, *878*, 1647.
29. Kashiwaba, N.; Ono, M.; Toda, J.; Suzuki, H.; Sano, T. *J. Nat. Prod.* **2000**, *63*, 477.
30. Tomita, M.; Watanabe, Y.; Furukawa, H. *Yakugaku Zasshi* **1965**, *85*, 77.
31. Ahmad, R.; Saa, J. M.; Cava, M. P. *J. Org. Chem.* **1977**, *42*, 1228.
32. Cuny, G. D. *Tetrahedron Lett.* **2003**, *44*, 8149.
33. Venkateswarlu, A.; Cava, M. P. *Tetrahedron: Asymmetry* **1976**, *32*, 2079.
34. Wu, H.; Liu, B.; Wang, W.; Shi, R. *Zhongcaoyao* **2010**, *41*, 514.
35. Zhang, Z.; ElSohly, H. N.; Jacob, M. R.; Pasco, D. S.; Walker, L. A.; Clark, A. M. *J. Nat. Prod.* **2002**, *65*, 856.
36. Tomita, M.; Watanabe, Y.; Furukawa, H. *Yakugaku Zasshi* **1961**, *81*, 1644.
37. Fajardo, V.; Araya, M.; Cuadra, P.; Oyarzun, A.; Gallardo, A.; Cueto, M.; Diaz-Marrero, A. R.; Darias, J.; Villarroel, L.; Álvarez, C.; Mora-Pérez, Y.; Joseph-Nathan, P. *J. Nat. Prod.* **2009**, *72*, 1355.
38. Markham, K. R.; Ternai, B.; Stanley, R.; Geiger, H.; Mabry, T. J. *Tetrahedron: Asymmetry* **1977**, *34*, 1389.
39. Manguro, L. O. A.; Ugi, I.; Lemmen, P.; Hermann, R. *Phytochemistry* **2003**, *64*, 891.
40. Lee, Y. S.; Lee, S.; Lee, H. S.; Kim, B.-K.; Ohuchi, K.; Shin, K. H. *Biol. Pharm. Bull.* **2005**, *28*, 916.
41. Lee, S.; Shin, D.-S.; Oh, K.-B.; Shin, K. H. *Arch. Pharm. Res.* **2003**, *26*, 40.
42. The ^{1}H and ^{13}C NMR spectra was assigned with the aid of distortionless enhancement by polarization transfer (DEPT), double quantum filter correlation spectroscopy (DQF COSY), heteronuclear multiple quantum coherence spectroscopy (HMQC), and heteronuclear multiple bond connectivity spectroscopy (HMBC) experiments.
43. The weak inhibitory activity of **8** seems to be attributable to its stability.
44. Lafrance, M.; Blaquière, N.; Fagnou, K. *Chem. Commun.* **2004**, 2874.
45. Shi, X.-X.; Ni, F.; Shang, H.-X.; Yan, M.-L.; Su, J. Q. *Tetrahedron: Asymmetry* **2006**, *17*, 2210.
46. Kunitomo, J.; Yamamoto, C.; Otsuki, T. *Yakugaku Zasshi* **1964**, *84*, 1100.
47. Si, Y.-G.; Gardner, M. P.; Tarazi, F. I.; Baldessarini, R. J.; Neumeyer, J. L. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3971.
48. Dothager, R. S.; Putt, K. S.; Allen, B. J.; Leslie, B. J.; Nesterenko, V.; Hergenrother, P. J. *J. Am. Chem. Soc.* **2005**, *127*, 8686.
49. Mach, U. R.; Hackling, A. E.; Perachon, S.; Ferry, S.; Wermuth, C. G.; Schwartz, J.-C.; Sokoloff, P.; Stark, H. *ChemBioChem* **2004**, *5*, 508.
50. Meuzelaar, G. J.; Maat, L.; Sheldon, R. A. *Tetrahedron: Asymmetry* **1999**, *55*, 4481.
51. Shinohara, T.; Takeda, A.; Toda, J.; Terasawa, N.; Sano, T. *Heterocycles* **1997**, *46*, 555.
52. Bertolotto, C.; Busca, R.; Abbe, P.; Bille, K.; Aberdam, E.; Ortonne, J.-P.; Ballotti, R. *Mol. Cell Biol.* **1998**, *18*, 694.