Tyrosinase Inhibitors from *Rhododendron collettianum* and Their Structure–Activity Relationship (SAR) Studies

Viqar Uddin AHMAD,* Farman ULLAH, Javid HUSSAIN, Umar FAROOQ, Muhammad ZUBAIR, Mahmud Tareq Hassan KHAN, and Muhammad Iqbal CHOUDHARY

H.E.J. Research Institute of Chemistry, University of Karachi; Karachi-75270, Pakistan.
Received April 1, 2004; accepted June 19, 2004

A new coumarinolignoid 8′-epi-cleomiscosin A (1) together with the new glycoside 8-*O*-β-D-glucopyranosyl-6-hydroxy-2-methyl-4*H*-1-benzopyrane-4-one (2) have been isolated from the aerial parts of *Rhododendron collettianum* and their structures determined on the basis of spectroscopic evidences. Tyrosinase inhibition study of these compounds and their structure–activity relationship (SAR) were also investigated. The compounds exhibited potent to mild inhibition activity against the enzyme. Especially, the compound 1 showed strong inhibition (IC_{50}=1.33 μM) against the enzyme tyrosinase, as compared to the standard tyrosinase inhibitors kojic acid (IC_{50}=16.67 μM) and L-mimosine (IC_{50}=3.68 μM), indicating its potential used for the treatment of hyperpigmentation associated with the high production of melanocytes.

Key words coumarinolignoid; methylchromone; *Rhododendron collettianum*; tyrosinase inhibition; structure–activity relationship

Rhododendron collettianum (Ericaceae) is widely distributed in Kurram Agency, Pakistan and the adjoining area in Afghanistan from 2000—3000 m. Some species of the genus *Rhododendron* have been used as medicinal plants. The dried leaves of *R. dauricum* are used medicinally as an expectorant and treatment of acute-chromic bronchitis.[1,2] Both flowers and fruits of *R. molle* have been recorded in ancient and modern monographs as analgesics in traditional uses. The chemical compound rhodojaponin isolated from *R. molle* has been shown to have significant blood pressure lowering and heart rate slowing effects.[3] The ethanolic extract of *R. arboreum* possesses hypotensive effect and respiratory stimulant in cats and dogs. It is also reported to produce contraction of guines pig ileum and have CNS effect.[4] The methanolic extract of the leaves and twigs of *R. dauricum* was found to display significant anti-HIV activity.[5]

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing enzyme widely distributed in plants and animals. It catalyses the oxidation of monophenols, *o*-diphenols and *o*-quinones. Tyrosinase is known to be a key enzyme for melanin biosynthesis in plants and animals. Tyrosinase inhibitors, can therefore be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation. They also find uses in cosmetics for whitening and depigmentation after sunburn. In addition, tyrosinase is known to be involved in the molting process of insect and adhesion of marine organisms.[6]

The medicinal properties assigned to *Rhododendron* prompted us to investigate *Rhododendron collettianum* for its chemical constituents and their effects against the enzyme tyrosinase. According to the best of our knowledge, the chemical constituents of this plant have not been investigated so far. Now we report the isolation and structure elucidation of two new compounds 8′-epi-cleomiscosin A (**1**) and 8-*O*-β-D-glucopyranosyl-6-hydroxy-2-methyl-4*H*-1-benzopyrane-4-one (**2**). In addition, three known compounds cleomiscosin A (**3**), aquillochin (**4**), and 5,6,7-trimethoxycoumarin (**5**) are also reported for the first time from this plant.[7—9]

Results and Discussion

The concentrated chloroform extract of *Rhododendron collettianum* was chromatographed over silica gel and subsequent elution with 40% EtOAc–hexane afforded a three compound mixture of coumarinolignoids. The components were separated by preparative TLC over silica gel developed with EtOAc–hexane (40 : 60). Among the three compounds the most polar one was separated and characterized as 8′-epi-cleomiscosin A (**1**). The compound of medium polarity aquillochin (**4**),[8] and the least polar one cleomiscosin A (**3**),[7] were also separated and characterized. The present paper describes the isolation of 8′-epi-cleomiscosin A (**1**) and 8-*O*-β-D-glucopyranosyl-6-hydroxy-2-methyl-4*H*-1-benzopyrane-4-one (**2**).

8′-Epi-cleomiscosin A (**1**) was isolated as an amorphous powder. The molecular formula was determined as $C_{20}H_{18}O_8$, by an $[M+H]^+$ peak at *m*/*z* 387 in HR-FAB-MS spectrometry. 8′-Epi-cleomiscosin A (**1**) and cleomiscosin A (**3**)[7] showed striking resemblance in spectral data indicating a close similarity between the two molecules. Thus the ^{13}C-NMR spectrum of 8′-epi-cleomiscosin A (**1**) disclosed the presence of seven aliphatic carbons (–CH_2O–×1, CH_3–O×2, –CHO–×2, –CH=CH–O×1), twelve aromatic carbons (CH×4, C×2, C–O×6), and one carbonyl carbon like its congener cleomiscosin A (**3**).[7]

This was supported by the presence of two characteristic doublets of the coumarin methine proton H-3 at δ 6.38 and H-4 at δ 7.71 with the coupling constant of (J=9.4 Hz) in the

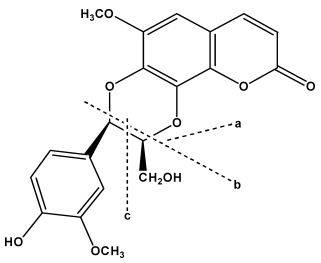

Fig. 1. Mass Fragmentation Pattern of the Compound **1**

* To whom correspondence should be addressed. e-mail: vuahmad@cyber.net.pk

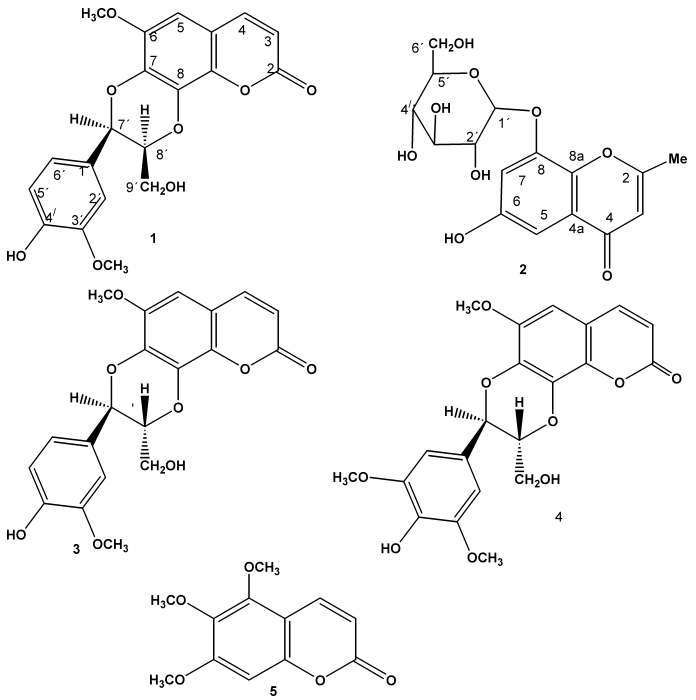

Fig. 2. Structures of the Compounds **1**—**5** Isolated from *Rhododendron collettianum*

^{1}H-NMR spectrum. Additional feature could be inferred from the chemical shifts and splitting pattern of its ^{1}H-NMR spectrum. Three aromatic methines gave an ABX pattern, in which the hydrogen at δ 7.41 appeared as a doublet (J=1.9 Hz) another hydrogen appeared as double doublet at δ 7.35 (J=8.1, 1.9 Hz) while the signal at δ 7.27 was doublet (J=8.1 Hz). The spectrum further showed two aromatic methoxyl groups at δ 3.80 and 3.68 each (3H, s). The signals at δ 5.55, 4.51 and 4.26 in the ^{1}H-NMR spectrum were found to be consistent with the following sequence –CHO–, –CHO–, and –CH_2–OH. As usual, the mass fragmentation pattern of the compound established the substitution pattern in both coumarin and the phenyl propane moieties. The presence of fragment ion at *m*/*z* 208 for $C_{10}H_8O_5$ in the mass spectrum of the compound indicated the position of the methoxy function in the coumarin nucleus. Similarly the genesis of the ion b at *m*/*z* 180 for $C_{10}H_{12}O_3$ and c at *m*/*z* 137 for $C_8H_9O_2$ could be explained by placing the second methoxyl function and the hydroxyl group at the phenyl propane system.

The ^{1}H- and ^{13}C-NMR data of 8′-epi-cleomiscosin A (**1**) are very similar to those of cleomiscosin A (**3**),[7] which was also isolated by us from the same source. However, one main difference was apparent. The cross peaks between H-7′/H-8′ in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum, indicated that these protons lie on one face of the molecule. No such cross peak between H-7′/H-8′ was observed in the NOESY spectrum of cleomiscosin A (**3**). This was further supported by the slightly downfield shift of ^{1}H- and ^{13}C-NMR at H-8′ (δ 4.52), C-8′ (80.5) of (**1**) as to (**3**) H-8′ (δ 4.34), C-8′ (79.5). However, in view of the optical activity $[\alpha]_D^{25}$=15.5. (c=0.1, C_5D_5N) of compound, **1** was concluded to be a single enantiomer, although some compounds of this type of skeleton were previously reported in the form of a racemic mixture.[7] The position of the methoxyl group and hydroxyl groups were also confirmed by heteronuclear multiple bond correlation (HMBC) and phenolic group at C-7 could also be inferred through HMBC correlations. The structure of compound **1** was finally assigned by selective heteronuclear decouplings.[7] Accordingly compound **1** was assigned the structure of 8′-epi-cleomiscosin A (**1**).

Table 1. NMR Data of Compound **1** (C_5D_5N)

Position	^{1}H	^{13}C	HMBC correlations
2		161.5 (s)	
3	6.38 (1H, d, J=9.4 Hz)	114.1 (d)	C-2, C-10
4	7.71 (1H, d, J=9.4 Hz)	144.5 (d)	C-2, C-9, C-5
5	6.72 (H, s)	101.5 (d)	C-9, C-4, C-6
6		146.5 (s)	
7		139.0 (s)	
8		132.7 (s)	
9		139.6 (s)	
10		112.5 (s)	
1′		127.2 (s)	
2′	7.41 (1H, d, J=1.9 Hz)	113.0 (d)	C-7′, C-3′, C-6′
3′		149.3 (s)	
4′		148.1 (s)	
5′	7.27 (1H, d, J=8.1 Hz)	117.3 (d)	C-3′, C-6′
6′	7.35 (1H, dd, J=8.1, 1.9 Hz)	122.1 (d)	C-1′, C-4′
7′	5.55 (1H, d, J=8.0 Hz)	77.5 (d)	C-8′, C-2′
8′	4.51 (1H, d t like (J=7.8 Hz))	80.5 (d)	C-7′
9′	4.26 (2H, br d, J=12.5 Hz)	61.5 (d)	C-7′
OMe	3.68 (3H, s)	56.0	C-3′
OMe	3.80 (3H, s)	56.1	C-6′

8-*O*-β-D-Glucopyranosyl-6-hydroxy-2-methyl-4*H*-1-benzopyrane-4-one (**2**) was obtained as a white powder and deduced to have the molecular formula $C_{16}H_{18}O_9$. The mass spectrum exhibited an $[M]^+$ peak at *m*/*z* 354 and an aglycone peak at *m*/*z* 192 formed by the loss of hexose. It was also conformed by its fast atom bombardments (FAB) mass spectrometry showing $[M+H]^+$ ion at *m*/*z* 355. The IR absorp-

Table 2. NMR Data of Compound **2** (MeOH)

Position	^{1}H	^{13}C	HMBC correlations
Me-2	2.38 (3H, s)	20.3 (q)	C-2, C-3
2		169.9 (s)	
3	6.11 (1H, s)	109.3 (d)	C-2
4		184.0 (s)	
4a		119.0 (s)	
5	6.6 (1H, d, J=2.0 Hz)	96.0 (d)	C-6, C-7, C-4
6		162.9 (s)	
7	6.4 (1H, d, J=2.1 Hz)	101.0 (d)	C-6, C-8, C-5
8		164.7 (s)	
8a		159.3 (s)	
1′	5.0 (1H, d, J=7.3 Hz)	101.6 (d)	C-8
2′	3.41 (m)	74.7 (d)	
3′	3.52′ (m)	78.3 (d)	
4′	3.53 (m)	71.4 (d)	
5′	3.41 (m)	77.8 (d)	
6′a	3.91 (dd, J=12.0, $J_{6,5}$=1.9 Hz)	62.4 (t)	
6′b	3.87 (dd, $J_{6b,6a}$=12.0, $J_{6,5}$=1.9 Hz)	62.4 (t)	

tion bands at 3390, 1710, 1573, and 1452 cm^{-1} indicated the presence of hydroxyl group, conjugated carbonyl, and aromatic ring respectively. Moreover the ^{1}H-NMR spectrum showed signal for the aromatic protons as doublets of protons at *meta* position, one olefinic proton, one glycosyl anomeric proton and one methyl group on a double bond. After acid hydrolysis of the compound **2**, the liberated sugar was found to be glucose by comparison on TLC with an authentic sample. This was further, conformed by ^{13}C-NMR data (Table 2) which agreed well with data published for β-D-glucose.[12] And also by coupling constant (d, J=7.3 Hz) in the ^{1}H-NMR spectrum. Further from ^{1}H-NMR pattern it showed that the glucose is *meta* substituted with respect to hydroxyl group. However the position of the glucose was conformed by ultra violet (UV) absorption and HMBC correlation. There was no change in the UV maxima by the addition of $AlCl_3$. It suggested the absence of free OH group at C-5 position. Similarly no change was observed by the addition of NaOAc, which indicated the absence of OH group at C-7 position.[10,11] So according to these observations and ^{13}C-NMR data shown in Table 2, the compound **2** is 8-*O*-β-D-glucopyranosyl-6-hydroxy-2-methyl-4*H*-1-benzopyrane-4-one.

Tyrosinase inhibition studies on the compounds isolated from the *Rhododendron collettianum* was carried out. Compound **1** exhibited highly potent inhibition (IC_{50}=1.33 μM) against the enzyme tyrosinase, when it is compared with the standard tyrosinase inhibitors kojic acid (IC_{50}=16.67 μM) and L-mimosine (IC_{50}=3.68 μM). Interestingly, compound **3** showed moderate inhibition (IC_{50}=18.69 μM) against tyrosinase, where both the compounds are structurally very much similar. Only the difference between these two compounds is that the compound **1** contains an α proton at the position 8′, where as compound **3** contains a β proton at the same position. Due to the change of stereochemistry of a single proton drastically changes the inhibitory activity of the compounds. This may be due to the stereochemically favourable binding condition at the active site of the enzyme. The inhibition pattern of the compound **4** (IC_{50}=15.69 μM) is also similar like the **3**. Stereochemically also, these two (**3**, **4**) are similar. But the compound **4** was showing more potency than that of kojic acid (IC_{50}=16.67 μM).

Compound **2** exhibited very mild (IC_{50}=256.97 μM) inhibition against the enzyme. This may be due to the presence of the glucose moiety, which may be creating barrier to be closer to the enzyme's active site due to its bulky nature.

Table 3. Tyrosinase Inhibitory Activities of the Compounds from *Rhododendron collettianum*, as Compared with the Standard Inhibitors

Name of the compounds	IC_{50}±S.E.M.[a] (μM)
Compound **1**	1.33±1.059
Compound **2**	256.97±0.598
Compound **3**	18.69±0.678
Compound **4**	15.69±0.694
Compound **5**	8.65±0.951
Kojic acid[b]	16.67±0.519
L-Mimosine[b]	3.68±0.02234

a) S.E.M. is the standard error of the mean. *b*) are the standard inhibitors of the enzyme tyrosinase.

Compound **5** showed potent inhibition (IC_{50}=8.65 μM) against the enzyme tyrosinase, when it was compared with the standard tyrosinase inhibitors kojic acid (IC_{50}=16.67 μM), but moderate as compared to the L-mimosine (IC_{50}=3.68 μM).

From this study, it can be concluded that courmarines isolated from the plant *Rhododendron collettianum*, can be effective inhibitors of tyrosinase enzyme and have a potential to be used for the treatment of hyperpigmentation associated with the high production of melanocytes.

Experimental

General Column chromatography (CC): silica gel, 70—230 mesh. Flash chromatography (FC): silica gel 230—400 mesh. TLC: pre-coated silica gel G-25-UV254 plates: detection at 254 nm, and by ceric sulphate reagent. Optical rotation. Jasco-DIP-360 digital polarimeter. UV and IR Spectra: Hitachi-UV-3200 and Jasco-320-A spectrometers, respectively. ^{1}H- and ^{13}C-NMR, COSY, NOESY, HMBC and HMQC Spectra: Bruker spectrometers operating at 500 and 400 MHz, using TMS as internal standard, chemical shifts δ in ppm and coupling constants in Hz. EI-, CIMS: JMS-HX-110 with a data system.

Plant Material The aerial parts of the plant of *Rhododendron collettianum* was collected from Parachinar (Kuram Agency) Afghanistan border NWFP Pakistan in August 2003, and identified by Prof. M. Qaiser, Department of Botany, University of Karachi. A voucher specimen (KUGH, No. 68216) has been deposited in the herbarium of the Department of Botany, University of Karachi, Pakistan.

Extraction and Isolation The whole plant (20 kg) was extracted with MeOH (50 l) for 30 d yielding 1.6 kg extract. The extract was suspended in water (1 l) and extracted with *n*-hexane (15 l) to yield hexane fraction (700 g) and then extracted with $CHCl_3$ (10 l) to yield chloroform fraction (140 g) and similarly with EtOAc (10 l) to yield ethyl acetate fraction (300 g). Chloroform fraction was then subjected to column chromatography on silica gel eluting with *n*-hexane–$CHCl_3$, $CHCl_3$ and $CHCl_3$–MeOH in increasing order of polarity to obtain seven small fractions (A—G). The fraction B obtained from column chromatography *n*-hexane–$CHCl_3$ (4 : 6) was again subjected to column chromatography with a solvent gradient from (*n*-hexane–EtOAc) to afforded four fractions (B_1—B_4). The fraction B_1 obtained from *n*-hexane–EtOAc (9 : 1) was further purified by column chromatography using silica gel with *n*-hexane–EtOAc (8 : 2) to furnish a compound **5** (7 mg). The second fraction B_2 obtained from *n*-hexane–EtOAc (6 : 4) was rechromatographed using silica gel with *n*-hexane–EtOAc (5 : 5) which afforded three compounds. The least polar was **3**[6] (16 mg) medium polar (**4**)[7] (10.5 mg) and the most polar one was **1** (4.5 mg). The fraction E obtained from 100% $CHCl_3$ was also rechromatographed using silica gel with $CHCl_3$–MeOH (9 : 1) afforded compound **2** (13 mg).

Compound **1**: Amorphous powder ^{1}H-NMR (500 MHz), ^{13}C-NMR and HMBC correlation data are shown in the Table 1. IR ($CHCl_3$) ν_{max} 3380, 2923, 2852, 1710, 1648, 1614, 1573, 1518, 1452, 1416, 1150 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 329 (2.09), 219 (2.53), 214 (2.04). EI-MS *m*/*z* (rel. int.) 137 (86), 180 (144), 208 (10), 354 (2), 368 (6), 368 (2). HR-FAB-MS *m*/*z* 387.1076 $[M+H]^+$ (Calcd for $C_{20}H_{18}O_8$ 386.1001). $[\alpha]_D^{25}$=15.5° (c=0.18,

C_5D_5N).

Compound **2**: White powder ^{1}H-NMR (500 MHz, MeOH), ^{13}C-NMR (500 MHz) (MeOH) and HMBC correlation data are shown in the Table 2. IR ($CHCl_3$) ν_{max} 3390 2922, 2852, 1740, 1710, 1659, 1623, 1573, 1509, 1452, 1416, 1391, 1339, 1135 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 285 (3.02), 250 (3.46), 228, (3.44). EI-MS *m/z* (rel. int.) 192, (100%), $(M-162)^+$ 164 (10), 124 (6), 354 (1). HR-FAB-MS *m/z* 355.1027 $[M+H]^+$ (Calcd for $C_{16}H_{19}O_9$ 354.0950). $[\alpha]_D^{25}=-75.1°$ (c=0.056, MeOH).

Tyrosinase Inhibition Assay Tyrosinase inhibition assays were performed in 96-well microplate format using SpectraMax 340 microplate reader (Molecular Devices, CA, U.S.A.) according to the method developed by Hearing in (1987).[13] Briefly, first the compounds were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the active inhibitors from the preliminary screening were subjected to IC_{50} studies. Compounds were dissolved in methanol to a concentration of 2.5%. Thirty units of mushroom tyrosinase (28 nM from Sigma Chemical Co., U.S.A.) were preincubated with the compounds in 50 nM Na-phosphate buffer (pH 6.8) for 10 min at 25 °C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 °C) due to the formation of the DOPAchrome for 10 min. The percent inhibition of the enzyme was calculated as follows, by using MS Excel®™ 2000 (Microsoft Corp., U.S.A.) based program developed for this purpose:

$$\text{percent inhibition}=[ABS_{Blank}-ABS_{Sample}/ABS_{Blank}]\times 100$$

Here, the ABS_{Blank} is absorbance for the blank and ABS_{Sample} is absorbance for the samples. After screening of the compounds, median inhibitory concentration (IC_{50}) was also calculated. All the studies have been carried out at least in triplicates and the results represent the mean±S.E.M. (standard error of the mean). Kojic acid and L-mimosine were used as standard inhibitors for the tyrosinase and both of them were purchased from Sigma Chem. Co., U.S.A.

Acknowledgments One of us (M.T.H.K.) gratefully acknowledges the travel support from the Third World Academy of Sciences (TWAS), Italy, and to the LG Corporation Ltd., Pakistan, for financial support.

References

1) Jiangsa, "Chinese Materia Medica," New Medical College, Shanghai people's Pub. House, Shaghai, 1977, p. 2506.
2) Yasuda M., Igarashi H., Kano Y., Konoshima M. S., *Shoyakugaku Zasshi*, **38**, 346—354 (1984).
3) Li C.-J., Wang L.-Q., Chem S.-N., Qin G.-W., *J. Nat. Prod.*, **63**, 1214—1217 (2000).
4) Thesis for M. Pharm. (Pharmacology) on Photochemical and Pharmaceutical studies on the flowers of *Rhododendron erboreum*. University of Delhi, Sharma S. C., 1986.
5) Kashiwada Y., Yamazaki K., Ikeshiro Y., Yamagishi T., Fujioka J., Mihashi K., Mizuki K., Cosentino L. M., Fowke K., Morris-Natschke S. L., Kuo-Hsiung L., *Tetrahedron*, **57**, 1559—1563 (2001).
6) Shiino M., Watanabe Y., Umezawa K., *Bioorg. Med. Chem.*, **9**, 1233—1240 (2001).
7) Anil B. R., Snail K. C., *Heterocycles*, **19**, 19—22 (1982).
8) Bhandar P., Pant P., Rastogi R. P., *Pytochemistry*, **21**, 2147—2149 (1982).
9) Wagner H., Bladt S., *Phytochemistry*, **14**, 2061—2064 (1975).
10) Ghosal S., Singh S., Mahendra P., Bhaght Y., Yatcndra Kumar B., *Phytochemistry*, **21**, 2943—2946 (1982).
11) Mabry T. J., Martham K. R., Thoumas M. B., "The Systematic Identification of Flavonoids," Springer-Verlag, New York, 1970.
12) Seo S., Tomita Y., Tori K., Yashimura Y., *J. Am. Chem. Soc.*, **100**, 3331—3339 (1978).
13) Hearing V. J., Jr., *Methods Enzymol.*, **142**, 154—165 (1987).