# Tyrosinase Inhibitor Fatty Ester and a Quinoline Alkaloid from *Skimmia laureola*

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Aerial parts of *Skimmia laureola* yielded a new tyrosinase inhibitor fatty ester, (+)-skimmidiol (**1**), and a new alkaloid ribaliprenylene (**2**). The configuration at C-3' in **1** was established by Horeau's procedure. Compound **1** was screened for its enzyme inhibitory activity against tyrosinase (E.C.1.14.18.1), exhibiting activity with $IC_{50}$ $51.25 \pm 1.10165$ $\mu$M.

*Key words: Skimmia laureola*, Fatty Acid Ester, Tyrosinase Activity, Rutaceae

## Introduction

*Skimmia laureola* Hook (Rutaceae) is found in Kashmir and in the mountains of Northern Pakistan and is used in folklore medicine for the treatment of various ailments [1 – 3]. The quinoline alkaloids of this plant have demonstrated antifungal and immunomodulating properties [4, 5]. The aerial parts of *S. laureola* were collected from Kashmir (Pakistan). The ethanolic extracts of the aerial parts of *S. laureola* are active against the animal pathogen *Microsporium canis* and the plant pathogen *Fusarium solani var. lycopersici* (tomato) [5].

## Results and Discussion

(+)-Skimmidiol (**1**) was isolated as a yellow oil from the petroleum ether extract of *S. laureola* by column and thin-layer chromatography. The UV spectrum of **1** showed absorptions at 212, 234, 199 and 192 nm, characteristic for the benzene ring bearing ester compounds. The IR spectrum of **1** showed intense absorptions at 3420 (OH), 2850 (C-H), 1630 (C=C), 1136 and 1090 (C-O) $cm^{-1}$. The high resolution electron-impact mass spectrum of **1** gave a molecular ion at $m/z$ 462.3708, corresponding to the molecular formula $C_{29}H_{50}O_4$ and indicated the presence of five rings/double bonds. The ion at $m/z$ 419 is due to the loss of $C_3H_7$ (462-43), while the ion at $m/z$ 405 is due to the loss of $CH_2$ (419-14), indicating the presence of a butyl moiety. The linked scan mass spectrum of the molecular ion showed that the ion at $m/z$ 389 (fragment **a**) and 73 (fragment **b**) arose directly from the molecular ion, thereby confirming the presence of two hydroxyl groups on fragment **a** (Fig. 1). The ion at $m/z$ 107, (fragment **d**) corresponding to the formula $C_7H_7O$, may arise by the loss of ($C_{18}H_{34}O_2$) from the ion at $m/z$ 389 due to the formation of a hydroxytropylium ion, [2] confirming the presence of one OH group attached to the benzene ring.

The $^1$H NMR spectrum ($CDCl_3$, 500 MHz) of **1** showed 3H doublets at $\delta = 0.86$ ($J_{3,2} = 7.0$ Hz) and 0.88 ($J_{4,2} = 7.0$ Hz), which were assigned to the C-3 and C-4 methyl protons, respectively. Two protons have overlapping chemical shift ($\delta = 7.65$, $J_{21',22'} =$ 6 Hz, $J_{21',25'} = 3$ Hz) were assigned to H-21' and H-25'. The other two aromatic protons have overlapping chemical shift ($\delta = 7.44$, $J_{22',21'} = 6$ Hz, $J_{22',24'} =$ 4 Hz) were assigned to H-22' and H-24' respectively. Two double doublets at $\delta = 4.16$ ($J_{1a,2} = 8$ Hz, $J_{1b,2} =$ 6 Hz) and $\delta = 1.64$ ($J_{2,1a} = 8$ Hz, $J_{2,1b} = 6$ Hz) were due to the C-1 and C-2 aliphatic protons. Three multiplets resonating at $\delta = 1.21$, 1.25 and 1.38 were assigned to H-5', H-6' and H-19'. While a broad multiplet resonated at $\delta = 1.26 – 1.29$ integrating for 28 protons (H-17' to H-18') indicating the presence of an aliphatic methylene chain in the molecule. A comparison of $^1$H NMR chemical shifts of **1** with commiphotetrol was also done [6]. The absolute configuration at C-3' was determined by Horeau's method [7] to be *S*.

The $^{13}$C NMR broad-band spectrum ($CDCl_3$, 75 MHz) of **1** showed the resonances of twenty nine

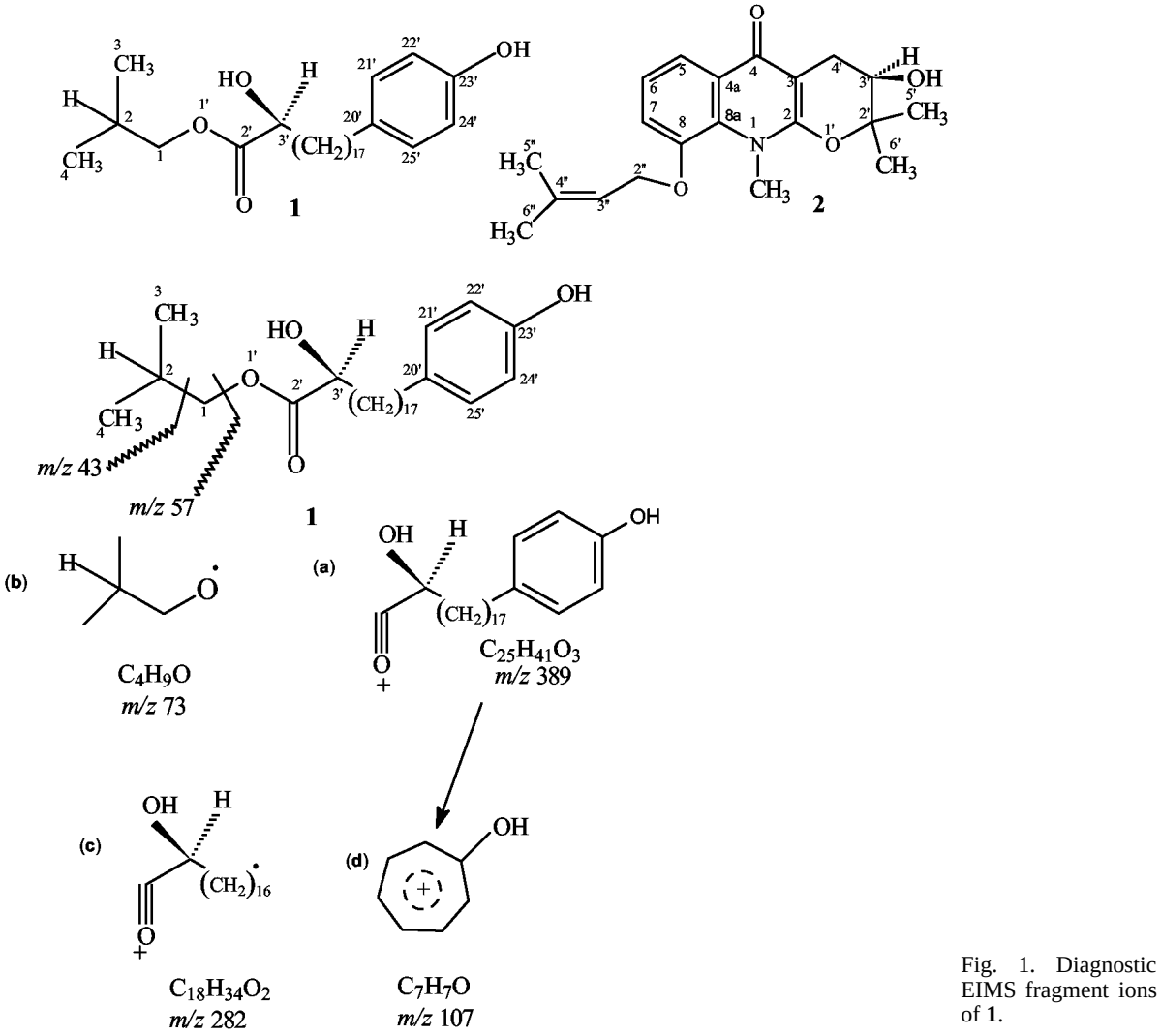



Fig. 1. Diagnostic EIMS fragment ions of **1**.

carbon atoms. Two carbons C-21'/C-25' have overlapping chemical shifts ($\delta = 128.77$). Similerly other two carbons C-22'/C-24' also have overlapping chemical shifts ($\delta = 130.76$). The DEPT spectra [8, 9] showed the presence of two methyl carbons, six methine and eighteen methylene carbons and hence, three quaternary carbons. The C-1 resonated at $\delta = 68.05$ ppm, and reflected its attachment to oxygen. The downfield carbon resonating at $\delta = 167.53$ was assigned to the C-2' carbonyl carbon. Two methyl carbons resonating at $\delta = 10.13$ and 13.11 were assigned to C-3 and C-4 carbons, respectively. Complete $^{1}H/^{13}C$ one-bond shift correlations of every protonated carbon is presented in Table 1.

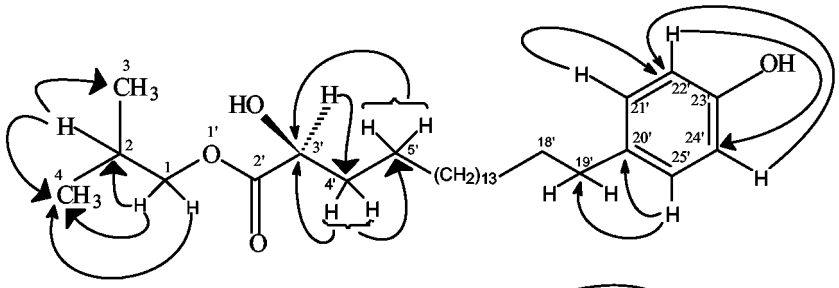
Two-dimensional NMR techniques such as COSY-45°, HMBC and HMQC [10] were used to provide further structural information. The one-bond $\Delta H/\Delta H$ couplings were established on the basis of COSY-45° and HOHAHA (20, 60, 100 ms) experiments. The assignment for H-2 ($\delta = 1.64$) could be confirmed by the cross-peak with H-3 ($\delta = 0.86$), H-4 ($\delta = 0.88$) and H-1 ($\delta = 4.16$) in the COSY-45° spectrum. Similarly the H-21' ($\delta = 7.65$) showed cross-peak with H-22' ($\delta = 7.44$) thereby, confirming the assignment of these vicinal protons to a benzene ring. The HOHAHA experiments together with the COSY results led to the existence of two main spin systems in **1**. The first spin system comprises

| Carbon Nos. | $^1$H NMR ($\delta$ ppm)[b] | Multiplicity[a] $J$ (Hz) | $^{13}$C NMR ($\delta$ ppm)[b] | Multiplicity[a] |
|---|---|---|---|---|
| 1 | 4.16 | dd, $J_{1a,2} = 8$ $J_{1b,2} = 6$ | 68.05 | $CH_2$ |
| 2 | 1.64 | dd, $J_{2,1a} = 8$ $J_{2,1b} = 6$ | 38.80 | -CH- |
| 3 | 0.86 | d, $J_{3,2} = 7.0$ | 10.13 | $CH_3$ |
| 4 | 0.88 | d, $J_{4,2} = 7.0$ | 13.11 | $CH_3$ |
| 2’ | – | – | 167.53 | -C- |
| 3’ | 3.60 | t | 77.60 | -CH- |
| 4’ | 2.24 | m | 31.90 | $CH_2$ |
| 5’ | 1.21 | m | 22.90 | $CH_2$ |
| 6’ | 1.25 | m | 24.00 | $CH_2$ |
| 7’ – 18’ | 1.26 – 1.29 | m | 28.9 – 29.7 | $CH_2$ |
| 19’ | 1.38 | m | 30.43 | $CH_2$ |
| 20’ | – | – | 132.59 | -C- |
| 21’ | 7.65 | dd, $J_{21',22'} = 6$ Hz $J_{21',25'} = 3$ Hz | 128.77 | -CH- |
| 22’ | 7.44 | dd, $J_{22',21'} = 6$ Hz $J_{22',24'} = 4$ Hz | 130.76 | -CH- |
| 23’ | – | – | 143.30 | -C- |
| 24’ | 7.44 | dd, $J_{24',25'} = 6$ Hz $J_{24',22'} = 4$ Hz | 130.76 | -CH- |
| 25’ | 7.65 | dd, $J_{25',24'} = 6$ Hz $J_{25',21'} = 3$ Hz | 128.77 | -CH- |

Table 1. $^1$H (500 MHz) and $^{13}$C NMR (75 MHz) data for compound **1**.

[a] Multiplicity assignments based on DEPT experiment; [b] one-bond heteronuclear correlations determined by HMQC experiment

a)




b)

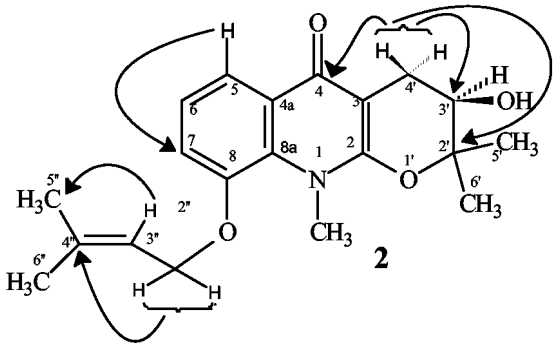



Fig. 2. a) Selected HMBC interactions for compound **1**. b) Selected HMBC interactions for compound **2**.

the four carbon protons *i.e.* H-1 ($\delta$ = 4.16), H-2 ($\delta$ = 1.64), H-3 ($\delta$ = 0.86) and H-4 ($\delta$ = 0.88). The second spin system consisted of protons C-3’ to C-19’.

The Heteronuclear Multiple Bond Connectivity (HMBC) [10] spectrum of **1** helped to determine the point of attachment of the alkyl chain on the benzene ring. In the HMBC spectrum, C-21’ and 25’ ($\delta$ = 128.77) showed long-range shift correlations with the methylene protons resonating at $\delta$ = 1.38 (H-19’). The protons of the C-19’ methylene carbon exhibited interactions with the methine carbon ($\delta$ = 132.59). Other important HMBC interactions are presented in Fig. 2a. These studies led to structure **1** for the compound. The results suggested that the compound under study had absolute configuration *S* at C-3’, being com-

posed of a 23 carbon chain having a benzene ring at C-19', attached by an ester link. Compound **1** exhibited *in vitro* tyrosinase (E.C.1.14.18.1) activity with $IC_{50}$ $51.25 \pm 1.10165$ $\mu$M. Kojic acid ($IC_{50} \pm$ S.E.M. $= 16.67 \pm 0.519$ $\mu$M) and L-mimosine ($IC_{50} \pm$ S.E.M. $= 3.68 \pm 0.02234$ $\mu$M) were used as a positive control [11–13].

Compound **2**, was isolated as a white powder. Its IR spectrum displayed strong absorptions at 3315 (OH),1706 (C=O) and 1632 (C=C) $cm^{-1}$. The UV spectrum of **2** showed absorptions at $\lambda_{max}$ (MeOH) 265 (2.67), 304 (3.84), 318 (3.86), 325 (3.74) and 330 (3.76) nm. The HREI MS spectrum of **2** showed the molecular ion peak at $m/z$ 343.1772, establishing the molecular formula as $C_{20}H_{25}O_4N$ and indicating nine double bond equivalents in the molecule. The ion at $m/z$ 275.1146 appeared due to the loss of $C_5H_8$ from the molecular ion.

The $^1$H NMR spectrum ($D_2O$, 500 MHz) of **2** showed the presence of five well separated singlets at $\delta = 3.94$, 1.38, 1.36, 1.82 and 1.76, which were assigned to $NCH_3$, *gem*-dimethyl C-5'/C-6' and C-5"/C-6" methyl protons, respectively. A downfield 1H double doublet at ($\delta = 8.33$, $J_{5,6} = 8.2$, $J_{5,7} = 1.1$ Hz), was assigned to H-5. The chemical shift of this proton indicated that the compound is a quinoline 4-one and not an isomeric quinoline 2-one derivative, since in the quinoline 4-one system, H-5 appears at about $\delta = 8.50$ [14]. A 1H double doublet ($\delta = 7.94$, $J_{6,5} = 8.2$ Hz, $J_{6,7} = 6.4$ Hz) was assigned to H-6, while another 1H double doublet at $\delta = 7.63$ ($J_{7,6} = 6.3$ Hz, $J_{7,5} = 1.8$ Hz) was assigned to H-7. The $^1$H NMR spectrum of 2 showed the downfield 1H doublet at $\delta = 4.24$ due to the C-3" methine proton. Two double doublets at $\delta = 4.63$, 4.53 were due to the C-2" methylene protons. Two 3H singlets at $\delta = 1.82$ and 1.76 were due to C-5" and C-6" protons. This indicated the presence of an isoprene group. The absence of aromatic proton at C-8 clearly indicated the attachment of an isoprene chain at C-8. Two 1H double doublets at $\delta = 3.57$ (dd, $J_{4'\beta,4'\alpha} = 11.2$ Hz, $J_{4'\beta,3'\alpha} = 4.8$ Hz) and 3.52 (dd, $J_{4'\alpha,4'\beta} = 11.2$ Hz, $J_{4'\alpha,3'\alpha} = 6.0$ Hz) were assigned to H-4'$\beta$ and H-4'$\alpha$ methylene protons. The C-3' methine proton appeared at $\delta = 3.64$ ($J_{3'\alpha,4'\alpha} = 6.0$, $J_{3'\alpha,4'\beta} = 1.0$ Hz), thus confirming the attachment of the hydroxyl group at C-3' instead of C-4' [15]. The chemical shifts of the C-4' methylene protons ($\delta = 3.52$ and 3.57) agreed with the attachment of the hydroxyl group at C-3' [16, 17] Table 2.

Table 2. $^1$H and $^{13}$C NMR chemical shift assignments for compound **2**.

| Carbon Nos. | $\delta^{13}$C | Multiplicity[a] | $\delta^1$H[b] ($J$ in Hz) |
|---|---|---|---|
| C-2 | 156.7 | (C) | – |
| C-3 | 93.3 | (C) | – |
| C-4 | 173.4 | (C) | – |
| C-4A | 139.1 | (C) | – |
| C-5 | 124.5 | (CH) | 8.33 (dd, $J_{5,6} = 8.2$, $J_{5,7} = 1.1$) |
| C-6 | 124.6 | (CH) | 7.94 (dd, $J_{6,5} = 8.2$, $J_{6,7} = 6.4$) |
| C-7 | 134.7 | (CH) | 7.63 (dd, $J_{7,6} = 6.3$, $J_{7,5} = 1.8$) |
| C-8 | 158.0 | (C) | – |
| C-8A | 126.3 | (C) | – |
| C-2' | 87.1 | (C) | – |
| C-3' | 67.7 | (CH) | 3.64 (dd, $J_{3'\alpha,4'\alpha} = 6.0$, $J_{3'\alpha,4'\beta} = 1.0$) |
| C-4' | 27.5 | ($CH_2$) | 3.52 (dd, $J_{4'\alpha,4'\beta} = 11.2$, $J_{4'\alpha,3'\alpha} = 6.0$), 3.57 (dd, $J_{4'\beta,4'\alpha} = 11.2$, $J_{4'\beta,3'\alpha} = 4.8$) |
| C-5' | 22.5 | ($CH_3$) | 1.38 (s) |
| C-6' | 20.8 | ($CH_3$) | 1.36 (s) |
| C-2" | 61.5 | ($CH_2$) | 4.63 (dd, $J_{2''\alpha,2''\beta} = 10.8$, $J_{2''\alpha,3''\alpha} = 8.4$), 4.53 (dd, $J_{2''\beta,2''\alpha} = 10.1$, $J_{2''\beta,3''\alpha} = 2.2$) |
| C-3" | 118.2 | (CH) | 4.24 (d, $J_{3"\beta,2"\alpha} = 8.0$) |
| C-4" | 139.1 | (C) | – |
| C-5" | 14.4 | ($CH_3$) | 1.82 (s) |
| C-6" | 25.1 | ($CH_3$) | 1.76 (s) |
| $NCH_3$ | 32.0 | ($N\underline{C}H_3$) | 3.94 (s) |

[a] Multiplicity assignments based on DEPT experiment; [b] one-bond heteronuclear correlations determined by HMQC experiment.

The $^{13}$C NMR spectrum of **2** ($D_2O$, 125 MHz) indicated the presence of 22 carbons. DEPT spectrum showed two methylene, five methine, five methyl and (by difference) eight quaternary carbon atoms in the molecule. Three oxygen-bearing carbons resonating at $\delta = 156.7$, 173.4 and 158.0 were assigned to C-2, C-4 and C-8 carbons, respectively. The signal at $\delta = 67.7$ was assigned to the hydroxy bearing C-3' methine carbon, which agrees with the proposed structure. Comparison of the $^{13}$C NMR chemical shifts of C-2 and C-3 of compound **2** with (*R*)-(+)-ribalinine (**6**) and (+)-(*S*)-$\psi$-ribalinine(**5**) structurally agreed [15, 16]. Three methine carbons resonating at $\delta = 124.5$, 124.6 and 134.7 were assigned to C-5, C-6 and C-7, respectively. This tallied with the chemical shifts of the corresponding aromatic carbons of ravenine and oligophyline [18]. The $^{13}$C NMR spectrum of **2** showed the presence of one methylene carbon ($\delta = 61.5$), a methine carbon ($\delta = 118.2$), two methyls ($\delta = 14.4$, 25.1) and a quaternary carbon ($\delta = 139.1$) in close proximity, confirming the presence of an isopentenyl group. On the basis of these spectroscopic studies, **2** was assigned the structure ribaliprenylene. The $^1$H and $^{13}$C NMR spectra of **2** were found to be consistent with the proposed structure (Table 2).

The Heteronuclear Multiple Quantum Coherence (HMQC) [10] spectrum of **2** displayed cross peaks between the directly coupled carbon-proton pairs. The Heteronuclear Multiple Bond Connectivity (HMBC) [10] spectrum of **2** helped to determine the point of attachment of the isopentenyl alkyl chain at C-8. Moreover, C-8 ($\delta = 158.0$) showed long-range interactions with H-2" ($\delta = 4.63$, 4.53). Other HMBC interactions are presented in Fig. 2b. These studies led to structure **2**.

## Experimental Section

*General experimental procedures.* The mass spectra were recorded on a Jeol HX-110 instrument. The $^1$H and $^{13}$C NMR spectra were recorded in $CDCl_3$ at 500 and 400 and 125 and 75 MHz, respectively, on a Bruker AM-500, 400 NMR spectrometer. The UV and IR spectra were recorded on Shimadzu UV-240 and JASCO A-320 spectrophotometers, respectively. Optical rotations were measured on a polatronic D Polarimeter. The purity of the compounds was checked on TLC (Si-gel, Merck $PF_{254}$, 0.25 mm thickness). Melting points were determined in glass capillary tubes using a Buchi 535 and a Gallenkamp 30/MF-370 melting point apparatus.

*Plant material.* The aerial parts of *S. laureola*, Hook (20 kg) were collected from Hazara in December. A voucher specimen (KUH #58106) was deposited in the Herbarium of Department of Botany, University of Karachi.

*Extraction and isolation.* Air-dried aerial parts of *S. laureola* (20 kg dry weight) were dried and extracted with EtOH (100 l). The EtOH extract was concentrated to a gum (822 g), dissolved in distilled water and extracted thoroughly with petroleum ether (45 l). The petroleum ether soluble portion was evaporated under reduced pressure to yield a gum (66.92 g), which was chromatographed on a Si-gel column (Merck, 70 – 230 mesh, 2025 g). The elution of the column was initiated with petroleum ether. The combined column sub-fractions 1 – 8 (5.91 g) obtained by elution with 1 : 9 acetone-petroleum ether, which showed similar TLC behaviour upon spraying with ceric sulphate reagent, were combined and again subjected to CC using Silica gel (type 60, 70 – 230 mesh, 200 g) and the column was eluted with petroleum ether : acetone (9 : 1). The sub-fractions obtained on elution of the column with *n*-hexane: acetone (20 : 80) were compared on TLC. Fractions 7 – 18 showing similar behaviour on TLC were combined and further purified by preparative TLC (Merck $PF_{254}$, 0.2 mm) using $CHCl_3$ as the eluent to afford pure Dragendorff's active ribaliprenylene (**2**) (28 mg, $1.4 \cdot 10^{-4}$% yield). The remaining aqueous layer was acidified with acetic acid to pH 3, then extracted with $CHCl_3$. The acidic layer was basified with $NH_4OH$ to pH 12 and extracted with $CHCl_3$ (40 l). The $CHCl_3$ soluble portion was dried as a crude alkaloidal mixture (74.96 g). The remaining aqueous layer was freeze dried (89 g) and chromatographed on a Si-gel column (70 – 230 mesh size, 2930 g). Fractions 30 – 35 (500 ml each) obtained on elution with 1 : 3 MeOH-$CHCl_3$ showed similar behaviour on TLC using ceric sulphate reagent, were combined (1 g) and subjected to CC (70 – 230 mesh, 32 g) eluting with 73 : 27 $CHCl_3$-MeOH. This afforded semi-pure fractions (29 – 45, 1 g), which were further purified by preparative TLC (Merck, $PF_{254}$, 0.2 mm) using 72 : 28 $CHCl_3$-MeOH to afford pure **1** (12.52 mg, $6.2 \cdot 10^{-5}$% yield, $R_f = 0.6$).

*(+)-Skimmidiol* (**1**): Yellow colored oil, $[\alpha]_D^{29} + 8$ (*c* 1, $CHCl_3$); UV/vis (MeOH): $\lambda_{max}(\log\varepsilon) = 234$ (3.01), 212 (3.01), 199 (3.84) nm. – IR ($CHCl_3$): $\nu_{max} = 3420$ (OH), 1136 (C-O), 1750 (C=O) cm$^{-1}$. – $^1$H NMR (500 MHz, $CDCl_3$): $\delta$: see Table 1. – $^{13}$C NMR (75 MHz, $CD_3OD$): $\delta$: see Table 1. – HREIMS, $m/z$ 57.0630 ($C_4H_9$), 444 ($C_{29}H_{48}O_3$).

*Ribaliprenylene* (**2**): Pale yellow gum, $[\alpha]_D^{29} + 10$ (*c* 1, $CHCl_3$), UV/vis (MeOH): $\lambda_{max}(\log\varepsilon) =$ 265 (2.67), 304 (3.84), 318 (3.86), 325 (3.74), 330 (3.76) nm. – IR ($CHCl_3$): $\nu_{max} = 3315$ (OH), 1706 (C=O),1120 (O-C) cm$^{-1}$. – $^1$H NMR (500 MHz, $D_2O$): see Table 2. – $^{13}$C NMR (125 MHz, $D_2O$): $\delta$: see Table 2. – HREIMS. $m/z$ 275.1274 (calcd. 272.1286, $C_{16}H_8NO_3$), 275.1146 (calcd. 275.1157, $C_{15}H_{17}NO_4$), F.D. MS: $m/z$ 343.

*Horeau's procedure*: The sample (5 mg, *ca*. 001 mmol) was added to a solution of racemic 2-phenylbutanoic anhydride (0.1 ml) in pyridine (0.5 ml). The resulting mixture was stirred overnight at room temperature. Distilled water (0.3 ml) was added and the reaction mixture allowed to stand for 30 min. NaOH (0.1 M) was added dropwise until pH 9 and then the solution was extracted with $CHCl_3$. The aqueous layer was acidified to pH 3 using HCl and the acidic layer extracted with benzene (10 ml). The benzene extract was evaporated to adjust the volume to 1 ml. The optical rotation of the resulting 2-phenyl butanoic acid in aqueous solution was positive (*R*), thereby establishing the *S*-configuration of the hydroxyl group at C-3' in **1**.

*Tyrosinase inhibition assay*: Tyrosinase inhibition assays were performed in a 96-well microplate format using a SpectraMax 340 microplate reader (Molecular Devices, CA, USA). First, the compounds were screened for *O*-diphenolase inhibitory activity of tyrosinase using L-DOPA as the substrate in a 96 well microplate using the method of Hearing [11]. All active inhibitors from preliminary screening were subjected to $IC_{50}$ studies. Compounds were dissolved in methanol, the final solvent mixture being 3.3%. Briefly, 30 units of mushroom tyrosinase (28 nM from Sigma Chemical Co., USA) was first 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 mix-

ture and the reaction 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, using MS Excel® 2000 (Microsoft Corp., USA) based program developed for this purpose. Then, after screening the compounds using the same program respective to the active compounds, the median inhibitory concentration ($IC_{50}$) was also calculated. All studies were at least triplicated and the results represent the Mean ± S.E.M. (standard error of the mean). Kojic acid ($IC_{50} \pm$ S.E.M. $= 16.67 \pm 0.519$ $\mu$M) and L-Mimosin ($IC_{50} \pm$ S.E.M. $= 3.68 \pm 0.02234$ $\mu$M) were used as standard inhibitors for the tyrosinase; both were purchased from Sigma Chem. Co., USA.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