Selagintamarlin A: A Selaginellin Analogue Possessing a 1H-2-Benzopyran Core from *Selaginella tamariscina*

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**ABSTRACT:** Selagintamarlin A (1), a novel selaginellin analogue featuring the unique motif of 1H-2-benzopyran, a new selaginpulvilin E (2), together with eight known analogues were isolated from *Selaginella tamariscina*. Their structures were elucidated by extensive spectroscopic analyses. A plausible biosynthetic pathway of 1 was also postulated. Compound 1 showed remarkable inhibitory activity against phosphodiesterase-4 (PDE4D2), with an IC$_{50}$ value of 40 nM, which is 20-fold higher than that of the positive control (rolipram). Furthermore, compound 1 significantly inhibited tubulin polymerization.

**INTRODUCTION**

Selaginellin represents a structurally intriguing class of phenolic pigments featuring unique alkynyl and $p$-quinone methide functionalities, which have hitherto been found only in the genus Selaginella. With their unique molecular architectures and compelling biological activities, selaginellin and its analogues have attracted great attention from the synthetic and pharmacological communities. Specifically, four newly discovered selaginellin analogues with a fluorene core, namely, selaginpulvins, separated from *S. pulvinata*, have been identified as natural inhibitors of PDE4D2. Phosphodiesterase-4 (PDE4), which is predominantly expressed in most inflammatory and immune cells, is a hydrolytic enzyme responsible for the degradation of cyclic adenosine monophosphate (cAMP) and has been identified as a therapeutic target in tumors, inflammatory diseases, and the central nervous system. Therefore, the search for new selaginellin analogues as inhibitors of PDE4 with powerful efficiency is fascinating.

*Selaginella tamariscina*, collected from Guangxi, has been widely used in traditional Chinese medicine for the treatment of hepatitis, hematuria, and traumatic injury. Research toward the discovery of novel PDE4 inhibitors from this plant afforded two new (1, 2) and eight known selaginellin analogues (3–10). To the best of our knowledge, 1 represents the first example of a selaginellin analogue characterized by a 1H-2-benzopyran core. Most of the isolates showed strong inhibitory activity against PDE4D2, and the most active, 1, significantly inhibited tubulin polymerization. In this article, the isolation, structural elucidation, plausible biosynthetic pathway, and biological evaluation of the isolated compounds are described.

**RESULTS AND DISCUSSION**

Selagintamarlin A (1) was obtained as a dark red powder. Its molecular formula, C$_{34}$H$_{24}$O$_{7}$, with 23$^\circ$ of unsaturation was established on the basis of negative-ion high-resolution electrospray ionization mass spectrometry (HRESIMS) (543.1464 [M − H]$^-$, calcd 543.1449) and $^{13}$C NMR data. Its UV spectrum showed absorption maxima at 216, 223, 260, 306, 388, and 428 nm, with characteristic values for the $\pi-\pi$ conjugate system of polyphenols. The IR spectrum showed absorption bands for hydroxyl (3433 cm$^{-1}$), carboxyl (1703 cm$^{-1}$), and aromatic ring (1609 and 1510 cm$^{-1}$) functionalities. The $^1$H NMR spectrum of 1 exhibited signals for an alkynyl [$\delta_H$ 7.40 (1H, s)], four $p$-substituted phenyl groups (two were superimposed) [$\delta_H$ 6.48 (4H, d, J = 8.8 Hz), 6.86 (4H, d, J = 8.8 Hz), 6.27 (2H, d, J = 8.8 Hz), 6.35 (2H, d, J = 8.8 Hz), 6.70 (2H, d, J = 8.8 Hz), and 7.49 (2H, d, J = 8.8 Hz)], and a 1,2,3,4-tetrasubstituted phenyl ring [$\delta_H$ 6.79 (1H, d, J = 8.0 Hz) and 7.82 (1H, d, J = 8.0 Hz)].

The $^{13}$C NMR spectrum of 1 showed 22 carbon signals, which disclosed the presence of a carboxyl (δ$_C$ 169.9), an alkynyl (δ$_C$ 153.8 and 98.3), four $p$-phenol groups (two were overlapped), a polysubstituted phenyl ring, and an unproto-
nated carbon (δC 89.2), with characteristic values of a selaginellin analogue.14−d Combining 1H and 13C NMR and HRESIMS data, the molecular formula of 1 was found to be C33H22O5; there should be one more ring in 1, as the five phenyl rings, one carboxyl, and one alkenyl only expended 22 of the 23° of unsaturation. The connections of these phenyl rings and other groups could be explained by two-dimensional NMR analyses. Strong heteronuclear multiple-bond correlations (HMBCs) from the aromatic protons at δH 6.86 (4H, d, H-3, 5, 8, 12) to the quaternary carbon (δC 89.2, C-7) indicate the presence of two geminal p-phenol groups at C-7. The alkenyl was linked to a p-phenol group by HMBCs from H-28 and H-32 to C-27, which was further placed at C-14 by HMBCs from H-26 to C-15 and C-19. The carboxyl and the last p-phenol group were located at C-15 and C-18 by HMBCs from H-16 to C-34 and H-20 and H-24 to C-18, respectively. The polysubstituted phenyl ring was further linked to C-7 by the observation of a weak J 4 HMBC from H-17 to C-7.7 On account of the structure of 1 demanding an additional ring, the remaining oxygen was linked to C-7 and C-27 to form a pyran ring. This was supported by the 13C NMR chemical shifts of C-7 and C-27, which were larger than normal. Therefore, according to the molecular composition of 1, a pyran ring attached by a polysubstituted phenyl ring to form a 1H-2-benzopyran core, as described in Figure 1, was suggested for 1.

Selaginulvin E (2) was obtained as a brown amorphous powder, and the molecular formula C13H12O2 was established by its negative-ion HRESIMS (497.1402 [M−H]+, calc 497.1394). The 1D NMR data of 2 were very similar to those of selaginulvins A−D (3−6),7 with the only difference due to the replacement of the hydroxymethyl group in selaginulvin A (3) by a hydroxyl group in 2. This was verified by the key HMBCs from H-17 (δH 7.49) to C-25 (δC 132.8) and H-16 (δH 6.86) and H-17 (δH 7.49) to C-15 (δC 158.0) (Figure 2).

The known compounds were identified as selaginulvins A−D (3−6),7 selaginellin A (7),15 selaginellin B (8),15 selaginellin E (9),15 and selaginellin O (10)14 by NMR spectroscopic analysis, as well as by the comparison of their physical and spectral data with those in the literature.

The structure of selagintamarlin A described here represents a hitherto unknown selaginellin skeleton. Thus, a putative biogenetic pathway for 1 is proposed (Scheme 1) to assist further studies for biomimetic semisynthesis or total chemical synthesis. The most plausible biosynthetic precursor of 1 is selaginellin O (10). Selaginellin O is oxidized to form intermediate I, and hydration of I would generate intermediate II12. The key step of this biosynthesis, the linkage of C7−OH and C-27 of intermediate II to afford 1, is probably due to the hydroxy group attacking the triple bond (shown as curved arrows in Scheme 1) to form a stable pyran ring.

All of the isolates were evaluated for their inhibitory activity against PDE4D2 and computational docking into the active site of PDE4D2. Compounds 1−10 potently inhibited the activity of PDE4D2, with IC50 values in the range of 40−1680 nM. In particular, 1, the most active compound, showed an IC50 of 40 nM, being 20-fold stronger than that of the positive control (Table 1 and Figure S9). The partition coefficient values of compounds 1−10 were calculated using the Marvin Sketch program, and the values (log D) of 1 at physiological pHs 3.0 and 7.4 were 6.86 and 3.60, respectively (Table S2). As shown in Figure 3a, compound 1, with the best docking score, was nicely bound to the active binding site and filled the cavity (Supporting Information S1).

Recent studies have suggested that PDE4 inhibitors have also been identified as a target agent for brain tumor therapy.8 Hence, compounds 1−10 were tested for antitumor activity against MGC-803, HepG2, A549, NCI-H460, and SKOV-3 human cancer cell lines using the MTT method, with fluorouracil as the positive control. The results are expressed as IC50 values, shown in Table S1. Most of the isolates exhibited moderate antitumor activity, especially compound 1 (IC50 values in the range of 7.02−10.32 μM).

It is worth noting that diarylacylonitrite (CC-5079) can act as a dual inhibitor of PDE4 and tubulin polymerization.13 Therefore, we also performed docking studies for compounds 1−10 to elucidate their structural basis. Surprisingly, the accommodation of compound 1 in the binding site is similar to that of colchicine, a well known tubulin polymerization inhibitor (Figure 3b,c). Furthermore, we investigated whether compound 1 acts as an inhibitor of tubulin polymerization in a dose-dependent manner.14 As shown in Figure 4, for compound 1, an obvious inhibition of polymerization was observed at two concentrations, and the rate of assembly as well as the final amount of microtubules was lower than that of the control.

In conclusion, selagintamarlin A (1) is a 1H-2-benzopyran selaginellin analogue with an unprecedented skeleton isolated.
Table 1. $^1$H and $^{13}$C NMR Data of 1 and 2 in CD$_3$OD ($\delta$ in ppm)$^a$

|   | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
|---|------------|------------|------------|------------|
| 1/10 | 157.7 | 156.8 | | |
| 2/6/9/11 | 6.48 (d, 8.8) | 114.6 | 6.58 (d, 8.8) | 115.1 |
| 3/5/8/12 | 6.86 (d, 8.8) | 132.3 | 7.09 (d, 8.8) | 131.4 |
| 4/13 | | 135.8 | 135.5 | |
| 7 | 89.2 | | 66.1 | |
| 14 | 135.9 | | 110.6 | |
| 15 | | 135.2 | | 158.0 |
| 16 | 7.82 (d, 8.0) | 130.4 | 6.86 (d, 8.0) | 115.1 |
| 17 | 6.79 (d, 8.0) | 130.9 | 7.49 (d, 8.8) | 120.4 |
| 18 | | 147.2 | | 134.2 |
| 19 | | 123.9 | | 154.9 |
| 20 | 6.35 (d, 8.8) | 129.8 | | 156.7 |
| 21 | 6.27 (d, 8.8) | 114.8 | 6.66 (d, 2.4) | 113.4 |
| 22 | | 156.3 | | 157.8 |
| 23 | 6.27 (d, 8.8) | 114.8 | 6.72 (dd, 8.4, 2.4) | 115.4 |
| 24 | 6.35 (d, 8.8) | 129.8 | 7.47 (d, 8.4) | 120.5 |
| 25 | | 135.4 | | 132.8 |
| 26 | 7.40 (s) | 98.3 | | 83.8 |
| 27 | | 153.8 | | 101.9 |
| 28/32 | 7.49 (d, 8.8) | 128.0 | 6.88 (d, 8.8) | 133.7 |
| 29/31 | 6.70 (d, 8.8) | 116.1 | 6.67 (d, 8.8) | 116.3 |
| 30 | | 159.9 | | 158.9 |
| 33 | | 127.2 | | 115.9 |
| 34 | | 169.9 | | |

$^a$H NMR recorded at 400 MHz; $^{13}$C NMR recorded at 100 MHz.

from S. tamariscina. Selaginpulvinil E (2) is an analogue of selaginpulvinil A–D. Their structures have been determined, and the formation of 1 and 2 was deduced from 10 and 7, respectively. The inhibitory activity of compounds 1–10 against PDE4D2 was deeply studied. Compound 1 showed remarkable inhibitory activity against PDE4D2 and tubulin polymerization, which indicated that 1 might be a dual inhibitor of PDE4D2 and tubulin polymerization.

■ EXPERIMENTAL SECTION

General Experimental Procedures. The NMR spectra were recorded in CD$_3$OD on a Bruker 400 MHz instrument (Bruker, Karlsruhe, Germany), with tetramethylsilane as the internal standard. HRESIMS data were obtained from a Thermo-Scientific Exactive spectrometer. IR spectra (KBr) were recorded on a PerkinElmer Spectrum Two FT-IR spectrometer. Silica gel (200–300 mesh; Qingdao Marine Chemical Factory, China), MCI gel (CHP20, 75–150 $\mu$m; Mitsubishi Chemical Corporation, Japan), ODS (50 $\mu$m; YMC, Japan), and Sephadex LH-20 gel (Pharmacia Biotech, Sweden) were used for column chromatography. Thin-layer chromatography was carried out on GF254 plates (Qingdao Marine Chemical Factory). Preparative high-performance liquid chromatography (HPLC) was carried out using a Shimadzu LC-6AD (Shimadzu, Japan) instrument with a YMC-Pack ODS-A column (20 mm I.D. × 250 mm, S-5 $\mu$m, 12 nm) and an SPD-20A wavelength detector at 210 nm. HPLC solvents were obtained from Tedia Company, Inc.

Plant Material. The herb of S. tamariscina was purchased from Jingxi County, Guangxi Zhuang Autonomous Region, in July 2014. The plant was authenticated by Prof. Shao-Qing Tang (Guangxi Normal University). A voucher specimen (No. 20140127) was deposited in the School of Life Sciences, Guangxi Normal University.

Extraction and Isolation. The dried whole herb of S. tamariscina (8,861 kg) was extracted with 95% EtOH at room temperature. The EtOH extract was concentrated under vacuum to give a crude extract (443 g). The crude extract was chromatographed on a silica gel column eluted with CH$_2$Cl$_2$/MeOH (50:1–1:1) to afford 19 fractions (F1 – F19).

Fraction 11 (4.3632 g) was separated by ODS CC, with gradient MeOH/H$_2$O (60–100%), to produce nine subfractions (F11a – F11i). A Sephadex LH-20 column using MeOH (100%) was prepared for F11f (417.2 mg), which finally gave four subfractions (F11f1 – F11f4). F11f1 (42.1 mg) was further purified on preparative HPLC (45% MeCN in H$_2$O, 8 mL/min) to yield 9 (17.7 mg, $t_R = 28.5$ min). F11f3 (34.5 mg) was depurated to acquire 10 (15.7 mg) directly. F11f4 (62.4 mg) was purified by preparative HPLC (45% MeCN in H$_2$O, 8 mL/min) to yield 1 (6.3 mg, $t_R = 36.0$ min). F11i (214.7 mg) was separated on Sephadex LH-20 gel using MeOH (100%) to afford 8 (24.9 mg).

Fraction 12 (20.313 g) was chromatographed over an MCI gel CC eluted with MeOH/H$_2$O (50–100%) to produce 20 subfractions (F12a – F12t). F12q (114.7 mg) was purified on preparative HPLC (70% MeOH in H$_2$O, 8 mL/min) to obtain 7 (24.4 mg, $t_R = 24.6$ min). 3 subfractions (F12m1 – F12m3) were obtained on a Sephadex LH-20 column using MeOH (100%) from F12m (347.0 mg). F12m1 (15.9 mg) was depurated by preparative HPLC (60% MeOH in H$_2$O, 8 mL/min) to afford 4 (6.3 mg, $t_R = 24.0$ min). F12m2 (40.4 mg) was purified by preparative HPLC (70% MeOH in H$_2$O, 8 mL/min) to yield 5 (17.1 mg, $t_R = 16.49$ min) and 6 (7.0 mg, $t_R = 21.76$ min).

Fraction 13 (10.4326 g) was subjected to MCI gel CC eluted with gradient MeOH/H$_2$O (50–80%) to produce 20 subfractions (F13a – F13t). F13k (40.5 mg) was separated by preparative HPLC (50% MeOH in H$_2$O, 8 mL/min) to yield 3 (24.0 mg, $t_R = 41.80$ min). F13m (26.2 mg) was purified by preparative HPLC (40% MeCN in H$_2$O, 8 mL/min) to obtain 2 (7.0 mg, $t_R = 27.6$ min). The purities of all compounds were above 92%, as determined by HPLC and NMR analysis.

Selagintamarlin A (1). Dark red powder; UV (c 0.01, MeOH) $\lambda_{\text{max}}$ 216, 223, 260, 306, 388, and 428 nm; IR $\nu_{\text{max}}$ (KBr) 3343, 1703, 1609, 1510, 1234, 1170, and 831 cm$^{-1}$; $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR data in CD$_3$OD, see Table 2; HRESIMS m/z 543.1464 [M + H]$^+$ (calcd for C$_{34}$H$_{24}$O$_7$, 543.1449).

Selaginpulvinil E (2). Brown amorphous powder; UV (c 0.01, MeOH) $\lambda_{\text{max}}$ 258, 287, 293, 300, 339, and 353 nm; IR $\nu_{\text{max}}$ (KBr) 3396, 1682, 1604, 1510, 1463, 1214, 830, and 529 cm$^{-1}$; $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR data in CD$_3$OD, see Table 2; HRESIMS m/z 497.1402 [M + H]$^+$ (calcd for C$_{33}$H$_{24}$O$_7$, 497.1394).

PDE4D2 Assay. Human recombinant (5 pg/µL) PDE4D2 (BPS Biosciences) was added to the wells of black 96-well nonbinding plates. Immediately, the protein was treated with the compound or vehicle control. The enzyme was incubated with the drug for 30 min prior to addition of 200 nM FAM-cAMP (Molecular Devices) to each assay well. The plates were incubated for 1 h at room temperature. After incubation, the terbium ( Tb)-based fluorescence intensity (FI) phosphodies- terase evaluation assay (Molecular Devices) binding reagent was added to each well, and the plates were incubated for an
additional 1 h at room temperature. FI was measured according to the manufacturer’s specifications using a TECAN plate reader. Each measurement was repeated at least three times. The IC$_{50}$ values were calculated by nonlinear regression. As a reference compound, rolipram purchased from Sigma was measured for its IC$_{50}$ value before the other assays.

**Tubulin Polymerization Assay in Vitro and Competitive Inhibition Assays.** Tubulin polymerization was monitored by the change in optical density at 340 nm using a modification of methods described by Jordan et al. A purified brain tubulin polymerization kit was purchased from Cytoskeleton (BK006P; Denver, CO). The final buffer concentrations for tubulin polymerization contained 80.0 mM piperazine-$N,N'$-bis(2-ethanesulfonic acid)disodium salt (pH 6.9), 2.0 mM MgCl$_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid, 1 mM guanosine-5'-triphosphate, and 10.2% glycerol. Test compounds were added in different concentrations, and then, all components except the purified tubulin were warmed to 37 °C. The reaction was initiated by the addition of tubulin to a final concentration of 3.0 mg/mL. Paclitaxel, CA-4, and phenstatin were used as positive controls under the same conditions. The optical density was measured for 1 h at 1 min intervals in BioTek’s Synergy 4 multifunction microplate spectrophotometer with a temperature-controlled cuvette holder. Assays were performed according to the manufacturer’s instructions and under conditions similar to those employed for the tubulin polymerization assays described above.

**Molecular Modeling.** All the docking studies were carried out using Sybyl-X 2.0 on a windows workstation. The crystal
structure of PDE4D2 and the tubulin in complex with colchicine (PDB: 3E22.pdb) were retrieved from the RCSB Protein Data Bank. The isolated compounds were selected for the docking studies. The three-dimensional structures of these selected compounds were first built using Sybyl-X 2.0 sketch, followed by energy minimization using the MMFF94 force field and Gasteiger-Marsili charges. We employed Powell’s method for optimizing the geometry with a distance-dependent dielectric constant and a termination energy gradient of 0.005 kcal/mol. All of the selected compounds were automatically docked into the colchicine-binding pocket of tubulin by an empirical scoring function and a patented search engine in the Surflex docking program. Before the docking process, the natural ligand was extracted; the water molecules were removed from the crystal structure. Subsequently, the protein was prepared using the Biopolymer module implemented in Sybyl. The polar hydrogen atoms were added. The automated docking manner was applied in the present work. Other parameters were established by default to estimate the binding affinity characterized by the Surflex-Dock scores in the software. Surflex-Dock total scores, which were expressed in \(-\log 10 (K_d)\) units to represent binding affinities, were applied to estimate the ligand–receptor interactions of the newly designed molecules. A higher score represents a stronger binding affinity. The optimal binding pose of the docked compounds was selected on the basis of the Surflex scores and visual inspection of the docked complexes.

Table 2. Enzymatic Activities (IC$_{50}$, nM) of 1–10 Against the Catalytic Domain of PDE4D2

| compd. | PDE4D2 (IC$_{50}$), nM | compd. | PDE4D2 (IC$_{50}$), nM |
|--------|----------------------|--------|----------------------|
| 1      | 40 ± 3.2             | 6      | 180 ± 14             |
| 2      | 190 ± 15             | 7      | 1680 ± 105           |
| 3      | 240 ± 40             | 8      | 140 ± 16             |
| 4      | 260 ± 17             | 9      | >10 000              |
| 5      | 110 ± 10             | 10     | 290 ± 21             |
| rolipram | 850 ± 76              |

![Figure 4](image-url) Effects of compound 1 on microtubule dynamics. Polymerization of tubulin at 37 °C in the presence of paclitaxel (10 μM), Combretastatin A4 (CA-4, 10 μM), and compound 1 (10 μM and 20 μM) was monitored continuously by recording the absorbance at 340 nm over 60 min. The reaction was initiated by the addition of tubulin to a final concentration of 3.0 mg/mL.

**ASSOCIATED CONTENT**

* Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00209.

Experimental procedures and IR, HRESIMS, and NMR spectra for 1 and 2 (PDF)

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**Author Contributions**

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**Notes**

The authors declare no competing financial interest.

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