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Selaginpulvilins A–D, New Phosphodiesterase-4 Inhibitors with an Unprecedented Skeleton from *Selaginella pulvinata*

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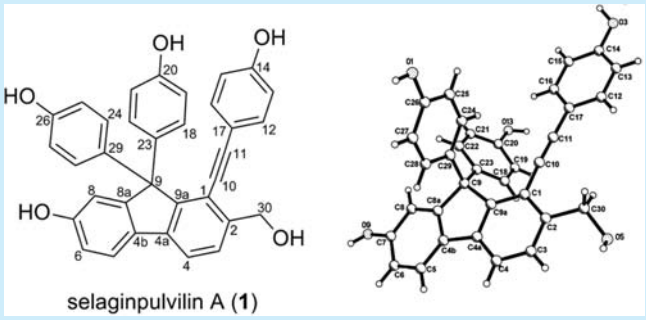
Supporting Information

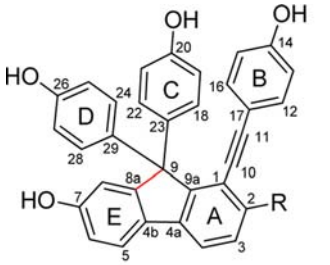
ABSTRACT: Selaginpulvilins A–D (**1**–**4**), four new phenols with an unprecedented 9,9-diphenyl-1-(phenylethynyl)-9*H*-fluorene skeleton, together with four known selaginellins (**5**–**8**) were isolated from *Selaginella pulvinata*. Their structures were elucidated by spectroscopic analysis and chemical correlation. The structure of **1** was confirmed by single-crystal X-ray diffraction. Compounds **1**–**8** exhibited remarkable inhibitory activities (IC_{50} values in the range of 0.11–5.13 μM) against phosphodiesterase-4 (PDE4), a drug target for the treatment of asthma and chronic obstructive pulmonary disease.



The phosphodiesterases (PDEs) are an 11-membered family of enzymes that catalyze the hydrolysis of the second messengers, cyclic adenosine monophosphate (cAMP) and guanosine monophosphate (cGMP).[1] Phosphodiesterase-4 (PDE4), which specifically targets cAMP, is a therapeutic target of high interest for central nervous system (CNS), inflammatory, and respiratory diseases.[2] Although a number of molecules of chemically diverse classes have been developed as PDE4 inhibitors over the decades, roflumilast is the sole PDE4 inhibitor recently approved in both the United States and Europe for the treatment of chronic obstructive pulmonary disease (COPD).[3] However, the efficacy of roflumilast may be restricted by the dose-limiting side effects of nausea, diarrhea, weight loss, and headaches. Thus, the search for novel PDE4 inhibitors with stronger potency and less side effects is compelling.

Selaginella pulvinata (Hook. et Grev.) Maxim. (Selaginellaceae), a qualified species listed in the Chinese Pharmacopoeia, has been widely used in Traditional Chinese Medicine (TCM) for the treatment of dysmenorrhea, asthma, and traumatic injury.[4] Previous investigations on this genus showed, in addition to flavonoids, the presence of 17 phenolic pigments featuring unique alkynyl and *p*-quinone methide functionalities.[5] In our efforts toward novel PDE4 inhibitors from medicinal plants, a fraction of the ethanolic extract of *S. pulvinata* showed significant inhibitory activity. Subsequent chemical investigation led to the isolation of four new phenols (**1**–**4**) with an unprecedented 9,9-diphenyl-1-(phenylethynyl)-9*H*-fluorene skeleton, together with four known selaginellins (**5**–**8**). Bioassay verified that compounds **1**–**8** had remarkable inhibitory activities against PDE4. Herein, the isolation, structural elucidation, biogenetic origin, and inhibitory activities of selaginpulvilins A–D are described.

The air-dried powder of the whole plant of *S. pulvinata* (1.0 kg) was extracted with 95% EtOH at room temperature to give a crude extract, which was suspended in H_2O and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. Column chromatographic separations of the EtOAc extract afforded compounds **1**–**8**.



1 R = CH_2OH **2** R = CHO
3 R = CH_3 **4** R = H

Selaginpulvilin A (**1**)[6] was obtained as a yellow crystal. The HRESIMS displayed a *pseudo*-molecular ion at *m*/*z* 511.1543 $[M - H]^-$ (calcd 511.1551), which was consistent with a molecular formula of $C_{34}H_{24}O_5$ with 23 degrees of unsaturation. The IR spectrum exhibited absorption bands for OH (3312 cm^{-1}), C≡C (2207 cm^{-1}), and benzene (1510 and 1607 cm^{-1}) functionalities. The ^1H NMR spectrum showed signals for three *p*-phenyl groups (two were overlapped) [δ_H 6.59 (4H, d, J = 8.0 Hz), 7.12 (4H, d, J = 8.0 Hz), 6.89 (2H, d, J = 8.3 Hz), and 6.71 (2H, d, J = 8.3 Hz)], a 1,2,4-trisubstituted benzene ring [δ_H 6.72 (1H, brs), 6.78 (1H, d, J = 8.0 Hz), and

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7.60 (1H, d, J = 8.0 Hz)], a 1,2,3,4-tetrasubstituted benzene ring [δ_H 7.49 (1H, d, J = 7.7 Hz) and 7.67 (1H, d, J = 7.7 Hz)], and a hydroxymethyl group [δ_H 4.80 (2H, s)]. The ^{13}C NMR spectrum of **1** exhibited 24 carbon resonances that were classified by DEPT experiments as two superimposed 4-hydroxylphenyl groups, a 4-hydroxylphenyl group, two multisubstituted benzene rings, an alkynyl (δ_C 85.4 and 102.4), an sp^3 quaternary carbon (δ_C 66.1), and a hydroxymethyl (δ_C 63.7). The aforementioned information implied that compound **1** possessed most structural features of selaginellin.[5a] However, as the five benzene rings and one alkynyl only consumed 22 of the 23 degrees of unsaturation, the remaining unsaturation unit required that **1** had one more ring than that of selaginellin.

The connectivities of these benzene rings, the quaternary carbon, and the alkynyl were mainly achieved by analysis of the HMBC correlations. Strong HMBC correlations from the aromatic protons at δ_H 7.12 (4H, d, H-18/22/24/28) to the quaternary carbon (δ_C 66.1, C-9) revealed the presence of two geminal 4-hydroxylphenyl groups at C-9. The HMBC correlations of H-5/C-4a and H-4/C-4b connected two multisubstituted benzene rings (A and E) via the C4b−C4a bond. Ring E was further linked to C-9 by HMBC correlation of H-8/C-9. The hydroxymethyl was located at C-2 by HMBC correlations from the protons at δ_H 4.80 (2H, s) to C-1, C-2, and C-3. The alkynyl was linked to a 4-hydroxylphenyl group by HMBC correlation of H-12 and H-16/C-11 to form a *p*-hydroxyl phenylethynyl, which was further placed at C-1 by a weak J^4 HMBC correlation from the hydroxymethyl to C-10. As an additional ring was required in the structure, the still "loose end" of C-9 and C-9a was linked to form a five-membered ring. This was supported by the observation of a weak J^4 HMBC correlation from H-18 to C-9a. Thus, a fluorene core attached by two *p*-phenyl groups at the central pentene ring and a *para*-pheylalkynyl at one of the benzene rings as depicted in Figure 1 was proposed for **1**, which was

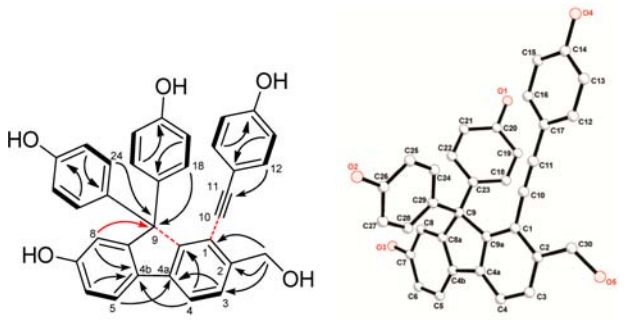


Figure 1. Key ^{1}H−^{1}H COSY (—) and HMBC (→) correlations of **1** (left). Single-crystal X-ray structure of **1** (right).

fully consistent with its molecular composition. Since the connectivities achieved by weak J^4 HMBC correlations remains ambiguous, a single-crystal X-ray diffraction analysis was carried out for **1**, which further secured the structure of **1**.[7]

Selaginpulvilin B (**2**),[8] isolated as yellow oil, had a molecular formula of $C_{34}H_{22}O_5$ as determined by HRESIMS at m/z 509.1386 [M − H]$^-$ (calcd 509.1394). The NMR data of **2** were very similar to those of **1**, with differences only attributable to the replacement of the hydroxymethyl group in **1** by a formyl group in **2** (δ_H 10.45, δ_C 192.9). This was further confirmed by HMBC correlations from the formyl proton to C-1, C-2, and C-3.

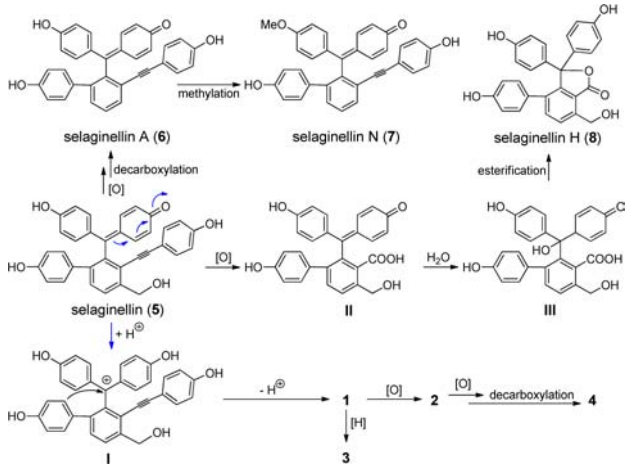
Selaginpulvilin C (**3**),[9] isolated as colorless oil, had a molecular formula of $C_{34}H_{24}O_4$ as determined by HRESIMS at m/z 495.1594 [M − H]$^-$ (calcd 495.1602). The NMR spectra of **3** were very similar to those of **1** except for the absence of signals for the hydroxymethyl group and the presence of a tertiary methyl (δ_H 2.43, δ_C 21.0), which indicated that the hydroxymethyl group in **1** was replaced by a methyl group in **3**. This was further confirmed by HMBC correlations from the methyl protons to C-1, C-2, and C-3.

Selaginpulvilin D (**4**),[10] isolated as colorless oil, had a molecular formula of $C_{33}H_{22}O_4$ as determined by HRESIMS at m/z 505.1417 [M + Na]$^+$ (calcd 505.1410). The ^{1}H and ^{13}C NMR data of **4** showed high similarity to those of **3** except that the methyl group in **3** was eliminated in **4**. This was supported by the characteristic 1,2,3-trisubstited pattern of ring A [δ_H 7.20 (d, J = 7.5 Hz), 7.28 (t, J = 7.5 Hz), and 7.65 (d, J = 7.5 Hz)] and further confirmed by detailed 2D analysis.

The known compounds were identified as selaginellin (**5**),[5a] selaginellin A (**6**),[5f] selaginellin N (**7**),[5b] and selaginellin H (**8**)[5g] by comparison of their NMR data with those in the literature.

Selaginpulvilins A−D (**1**−**4**) represent a new class of natural products possessing a fluorene core. Based on the co-occurrence of compounds **1**−**8**, the biogenetic pathway for them was proposed (Scheme 1). Selaginellin (**5**), the major

Scheme 1. Proposed Biosynthetic Pathway for Compounds 1−8



component, was considered as the precusor. In brief, **5** was modified via three different biosynthetic routes involving oxidative decarboxylation of hydroxymethyl, oxidative cleavage of alkynyl,[11] and acid-aided protonation to form selaginellin A (**6**), the intermediates **II**, and **I**, respectively. Methylation of **6** would generate **7**, and hydration of **II** followed by esterification could yield selaginellin H (**8**). The key step in this proposal, the linkage of the C-8a and C-9 of **I** to produce **1**, probably involved the aromatic electrophilic substitution reaction between ring E and a C-9 carbocation, similar to a Friedel−Crafts reaction. Finally, **1** underwent sequential oxidation to give **2** and **4** or underwent reduction to afford **3**.

To mimic the formation of the C8a−C9 bond, selaginellin (**5**) was reacted with different acids with the hope of generating **1** (Supporting Information S3). However, all efforts failed probably due to the vulnerability of the phenolic hydroxyl groups under acid condition. Thus, **5** was first converted to its

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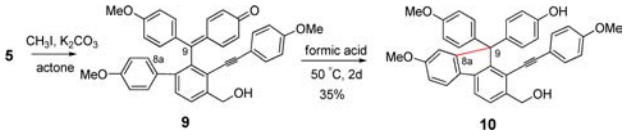
methylated derivative (**9**), which then reacted with formic acid at 50 °C in 2 days to successfully generate the C8a−C9 linked product, 7,14,26-trimethoxyselaginpulvilin A (**10**) (Scheme 2).

Scheme 2. Chemical Correlations to Mimic Formation of the C8a−C9 Bond



Compounds **1**−**8** were tested for their inhibitory activity against PDE4D2. Rolipram, a well-known PDE4 inhibitor, was used as the positive control, which exhibited an IC_{50} of 0.54 μM, comparable to that reported in the literature (1.0 μM).[1] Compounds **1**−**8** showed remarkable inhibitory activity with IC_{50} values in the range of 0.11−5.13 μM, which may explain the anti-inflammatory usage of *S. pulvinata* in TCM. In particular, selaginpulvilin B (**2**), the most active compound showed an IC_{50} of 0.11 μM, being 5-fold stronger than the positve control (Table 1). The dose−response curves of the two most active compounds (**2** and **3**) are represented in Figure 2. It was noteworthy that although compounds **5** and **6** were very active, their C8a−C9 connected counterparts, compounds **1** and **4**, showed at least 5-fold stronger inhibition, which suggested that formation of a fluorene core would enhance the activity.

Table 1. Enzymatic Activities (IC_{50}, μM) of Compounds 1−8 against the Catalytic Domain of PDE4D2

compound	IC_{50} (μM)
1	0.24 ± 0.03
2	0.11 ± 0.02
3	0.18 ± 0.02
4	0.26 ± 0.05
5	0.97 ± 0.10
6	1.42 ± 0.10
7	0.22 ± 0.01
8	5.13 ± 0.60
rolipram	0.54 ± 0.04

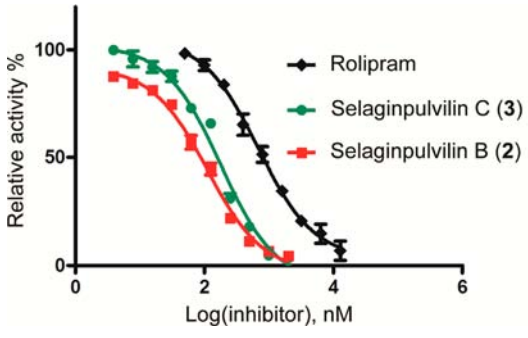


Figure 2. Inhibitory curves of compounds **2** and **3** against PDE4D2.

Natural PDE4 inhibitors are very rare. It is possible that the unique skeleton of selaginpulvins A−D renders them potent activity, which makes them promising lead structures for the development of PDE4 inhibitors. Studies toward their mechanism of action on PDE4 and their selectivity in the PDE family are in progress.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental procedures, tabulated NMR data of **1**−**4**, and 1D and 2D NMR spectra of **1**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(6) Selaginpulvilin A (**1**). Yellow crystal; mp 294−296 °C; UV (MeOH) λ_{max} (log ε) 204 (4.84), 289 (4.46), 300 (4.47), 320 (4.25) nm; IR (KBr) ν_{max} 3312, 2207, 1607, 1510, 1449, 1357, 1241, 1174, 833 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2 in Supporting Information; negative ESIMS *m/z* 511.2 $[M - H]^-$, 546.9 $[M + Cl]^-$, 556.7 $[M + HCOO]^-$; HRESIMS *m/z* 511.1543 $[M - H]^-$ (calcd for $C_{34}H_{23}O_5$, 511.1551).

(7) Crystallographic data for selaginpulvilin A (**1**) have been deposited at the Cambridge Crystallographic Data Centre (deposition no. CCDC-934814). Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.htm.

(8) Selaginpulvilin B (**2**). Yellow oil; UV (MeOH) λ_{max} (log ϵ) 208 (4.70), 294 (4.24), 305 (4.26), 314 (4.28), 360 (4.21) nm; IR (KBr) ν_{max} 3284, 2202, 1679, 1604, 1557, 1507, 1243, 1019 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2 in Supporting Information; positive ESIMS m/z 511.1 $[M + H]^+$, negative ESIMS m/z 509.1 $[M - H]^-$; HRESIMS m/z 509.1386 $[M - H]^-$ (calcd for $C_{34}H_{21}O_5$, 509.1394).

(9) Selaginpulvilin C (**3**). Colorless oil; UV (MeOH) λ_{max} (log ϵ) 208 (4.76), 288 (4.36), 299 (4.41), 315 (4.18) nm; IR (KBr) ν_{max} 3375, 2193, 1604, 1508, 1447, 1373, 1247, 1022, 829 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2 in Supporting Information; positive ESIMS m/z 497.2 $[M + H]^+$, negative ESIMS m/z 495.2 $[M - H]^-$; HRESIMS m/z 495.1594 $[M - H]^-$ (calcd for $C_{34}H_{23}O_4$, 495.1602).

(10) Selaginpulvilin D (**4**). Colorless oil; UV (MeOH) λ_{max} (log ϵ) 205 (4.89), 288 (4.52), 298 (4.53), 316 (4.35) nm; IR (KBr) ν_{max} 3367, 2203, 1563, 1509, 1468, 1170, 1073, 950, 863 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 1 and 2 in Supporting Information; positive ESIMS m/z 483.2 $[M + H]^+$, negative ESIMS m/z 481.2 $[M - H]^-$; HRESIMS m/z 505.1417 $[M + Na]^+$ (calcd for $C_{33}H_{22}O_4Na$, 505.1410).

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