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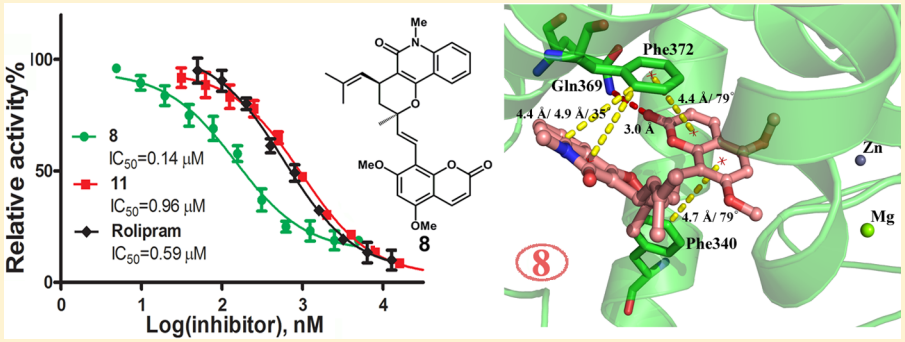
Prenylated Coumarins: Natural Phosphodiesterase-4 Inhibitors from *Toddalia asiatica*

Ting-Ting Lin,[†] Yi-You Huang,[†] Gui-Hua Tang, Zhong-Bin Cheng, Xin Liu, Hai-Bin Luo,* and Sheng Yin*

School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, Guangdong 510006, People's Republic of China

Supporting Information



ABSTRACT: Bioassay-guided fractionation of the ethanolic extract of the roots of *Toddalia asiatica* led to the isolation of seven new prenylated coumarins (**1**−**7**) and 14 known analogues (**8**−**21**). The structures of **1**−**7** were elucidated by spectroscopic analysis, and their absolute configurations were determined by combined chemical methods and chiral separation analysis. Compounds **1**−**5**, named toddalin A, 3‴-*O*-demethyltoddalin A, and toddalins B−D, represent an unusual group of phenylpropenoic acid-coupled prenylated coumarins. Compounds **1**−**21** and four modified analogues, **10a**, **11a**, **13a**, and **17a**, were screened by using tritium-labeled adenosine 3′,5′-cyclic monophosphate ([^{3}H]-cAMP) as substrate for their inhibitory activity against phosphodiesterase-4 (PDE4), which is a drug target for the treatment of asthma and chronic obstructive pulmonary disease. Compounds **3**, **8**, **10**, **10a**, **11**, **11a**, **12**, **13**, **17**, and **21** exhibited inhibition with IC_{50} values less than 10 μM. Toddacoumalone (**8**), the most active compound (IC_{50} = 0.14 μM), was more active than the positive control, rolipram (IC_{50} = 0.59 μM). In addition, the structure−activity relationship and possible inhibitory mechanism of the active compounds are also discussed.

The phosphodiesterases (PDEs) are an 11-membered family of enzymes that catalyze the hydrolysis of the secondary signal messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).[1] Phosphodiesterase-4 (PDE4), which specifically catalyzes the hydrolysis of cAMP, is a therapeutic target of high interest for central nervous system (CNS), inflammatory, and respiratory diseases.[2] Although a number of chemically diverse molecules have been developed as PDE4 inhibitors over the last 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] As the efficacy of roflumilast may be restricted by the dose-limiting side effects of nausea, diarrhea, weight loss, and headaches, the search for novel PDE4 inhibitors continues unabated.

Toddalia asiatica (L.) Lam. (Rutaceae), a woody climber, grows widely in south China. Its barks and roots have been extensively used in Traditional Chinese Medicine (TCM) for the treatment of rheumatic arthritis, traumatic injury, and pyogenic infections.[4,5] In the past decades, a number of prenylated coumarins and benzophenanthridine alkaloids have been isolated from this plant, some of which exhibited anti-inflammatory,[6] antiplatelet aggregation,[7] and antinitric oxide generation activities.[8]

In our continuing search for PDE4 inhibitors from medicinal plants, a fraction of the ethanolic extract of *T. asiatica* showed an inhibitory activity of 46.2% at a concentration of 10 μM by using tritium-labeled adenosine 3′,5′-cyclic monophosphate ([^{3}H]-cAMP) as the substrate toward PDE4. Subsequent chemical investigation led to the isolation of seven new prenylated coumarins (**1**−**7**) together with 14 known analogues (**8**−**21**). Compounds **1**−**5** represented an unusual group of phenylpropenoic acid-coupled prenylated coumarins. Compounds **1**−**21** together with four modified analogues, **10a**, **11a**, **13a**, and **17a**, were screened for their inhibitory activity against PDE4D2, and 10 compounds were identified as PDE4 inhibitors, with IC_{50} values ranging from 0.14 to 9.98 μM. Herein, details of the isolation, structural elucidation, inhibitory

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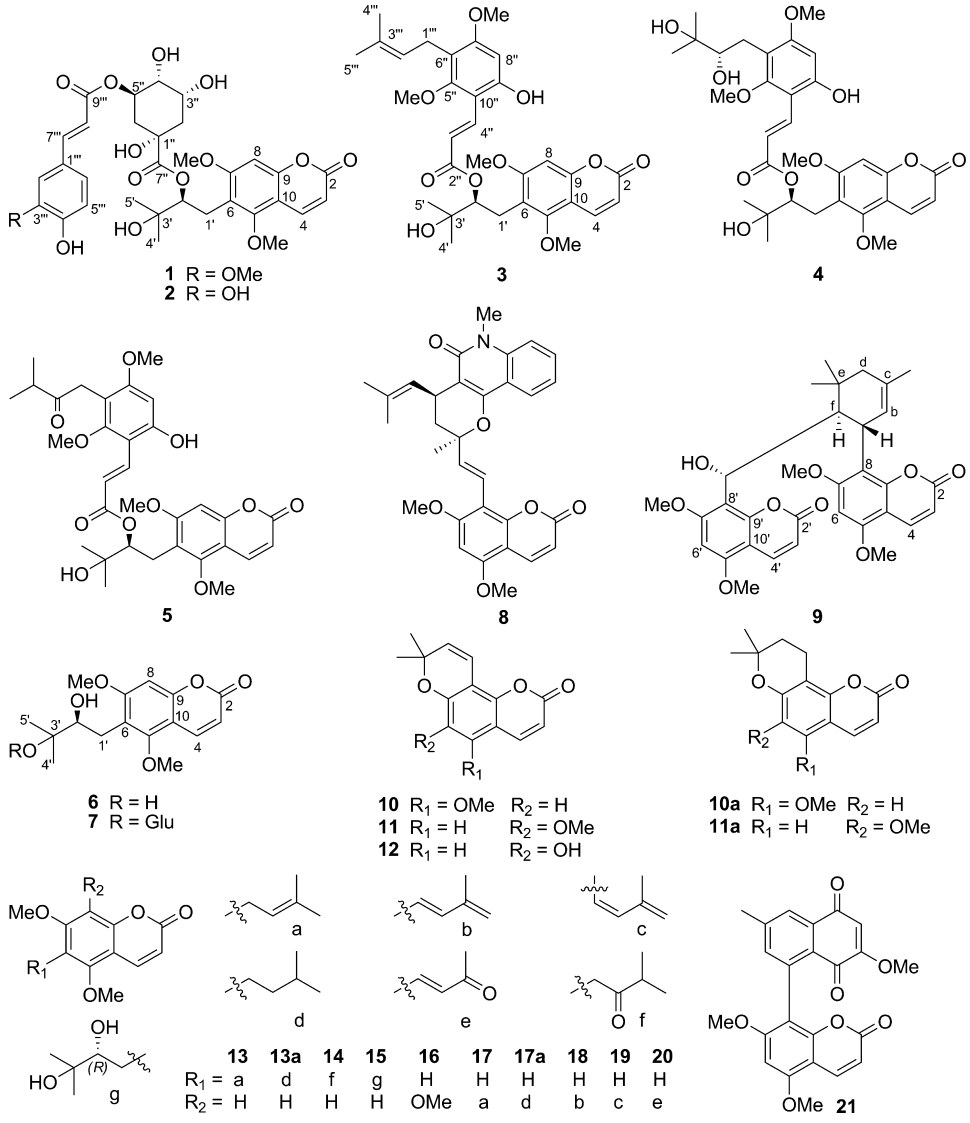
Chart 1



activities, structure–activity relationship, and possible inhibitory mechanism of these compounds are described.

RESULTS AND DISCUSSION

The air-dried powder of the roots of *T. asiatica* 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. Various column chromatographic separations of the EtOAc extract afforded compounds **1**–**21**.

Compound **6**, a white powder, was isolated as a major component by using HPLC equipped with a chiral column. The molecular formula of $C_{16}H_{20}O_6$ was determined by ^{13}C NMR data and the HRESIMS ion at *m*/*z* 331.1152 $[M + Na]^+$ (calcd 331.1158). The ^1H NMR spectrum of **6** comprising the prenylated coumarin features was identical to that of (+)-toddalolactone (**15**),[9] a major component previously reported from the same plant. However, the optical rotation of **6** ($[\alpha]_D^{20}$ −69) was opposite that of **15** ($[\alpha]_D^{20}$ +69),[10] indicating **6** was the enantiomer of **15**. This was supported by the co-injection of **6** and **15** on HPLC equipped with a chiral column, which gave two well-resolved peaks with different retention times. As the ^{13}C NMR data of **15** were not reported before, the full NMR spectrum of **6** was assigned in the current research. Thus, **6** was given the trivial name *ent*-toddalolactone.

Compound **1** exhibited a molecular formula of $C_{33}H_{38}O_{14}$ as determined by ^{13}C NMR data and the HRESIMS ion at *m*/*z* 657.2191 $[M - H]^-$ (calcd for $C_{33}H_{37}O_{14}$, 657.2189). The IR spectrum exhibited absorption bands for OH (3443 cm^{-1}), ester (1709 cm^{-1}), and benzene ring (1608, 1516, and 1458 cm^{-1}) functionalities. The ^1H NMR spectrum showed two methyl singlets [δ_H 1.24 (H_3-5′) and 1.31 (H_3-4′)], three methoxy groups (δ_H 3.86, 3.87, and 3.93), two *trans*-olefinic protons [δ_H 6.26 (1H, d, *J* = 16.0 Hz, H-8‴) and 7.57 (1H, d, *J* = 16.0 Hz, H-7‴)], two *cis*-olefinic protons [δ_H 6.12 (1H, d, *J* = 9.6 Hz, H-3) and 7.89 (1H, d, *J* = 9.6 Hz, H-4)], a 1,2,4-trisubstituted benzene ring [δ_H 6.82 (d, *J* = 8.2 Hz, H-5‴), 7.07 (d, *J* = 8.2 Hz, H-6‴), and 7.20 (s, H-2‴)], and four oxymethines [δ_H 3.57, 4.05, 5.21, and 5.28]. The ^{13}C NMR spectrum in combination with DEPT experiments resolved 33 carbon resonances attributable to three carbonyl carbons, two benzene rings, two vinylic groups, two oxygenated quaternary carbons, four oxygenated methines, three methoxy groups, three methylenes, and two methyls. The collective data implied

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that **1** comprised the structural features of ferulic and quinic acid moieties linked to compound **6**. Analysis of 2D NMR data permitted structure **1** to be proposed. In particular, HMBC correlations of H-5″/C-9‴ and H-2′/C-7″ confirmed the linkage of the three fragments. The structure of **1** was further confirmed by alkaline hydrolysis, which gave a mixture of three products. Ferulic and quinic acids were identified by co-TLC of the reaction mixture with authentic samples, while the coumarin was assigned as **6** by co-injection of the reaction mixture with **6** on HPLC equipped with a chiral column. Compound **1** was given the trivial name toddalin A.

Compound **2** displayed a molecular ion at *m/z* 667.1991 [M + Na]$^+$, consistent with a molecular formula of $C_{32}H_{36}O_{14}$, 14 mass units less than that of **1**. The ^{1}H and ^{13}C NMR data of **2** (Table 1) were similar to those of **1** except for the absence of a methoxy group, indicating **2** was a demethylated derivative of **1**. Analysis of 2D NMR data led to the proposal of structure of **2**, which revealed the replacement of the ferulic acid unit in **1** by a caffeic acid moiety in **2**. The absolute configuration of **2** was confirmed by using the same methods as described for **1**. Thus, **2** was given the trivial name 3‴-*O*-demethyltoddalin A.

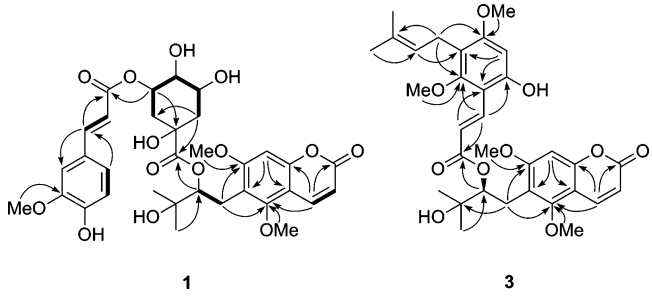
Compound **3** was assigned a molecular formula of $C_{32}H_{38}O_{10}$ as established on the basis of ^{13}C NMR data and the HRESIMS ion at *m/z* 605.2341 [M + Na]$^+$ (calcd 605.2357). The IR spectrum exhibited absorption bands for hydroxy (3445 cm^{-1}), ester (1709 cm^{-1}), and aromatic (1606, 1514, and 1456 cm^{-1}) functionalities. The ^{1}H NMR spectrum showed four methyl singlets [δ_H 1.34 (H_3-5′), 1.38 (H_3-4′), 1.64 (H_3-4‴), and 1.72 (H_3-5‴)], four methoxy groups [δ_H 3.59, 3.71, 3.80, and 3.84], two *trans*-olefinic protons [δ_H 6.65 (d, *J* = 16.0 Hz, H-3″) and 7.78 (d, *J* = 16.0 Hz, H-4″)], two *cis*-olefinic protons [δ_H 6.10 (d, *J* = 9.6 Hz, H-3) and 7.73 (d, *J* = 9.6 Hz, H-4)], two aromatic singlets [δ_H 6.21 (s, H-8″) and 6.49 (s, H-8)], and an olefinic proton [δ_H 5.09 (t, *J* = 6.4 Hz, H-2‴)]. The ^{13}C NMR spectrum, in combination with DEPT experiments, showed 32 carbon resonances attributable to two carbonyl carbons, two benzene rings, three double bonds, one oxygenated sp^3 quaternary carbon, one sp^3 oxymethine, four methoxy groups, two methylenes, and four methyls. The aforementioned data implied that **3** comprised the structural features of a prenylated cinnamic acid moiety linked to compound **6**. 2D NMR analysis allowed structure **3** to be postulated as depicted in Figure 1. In particular, HMBC correlations of H-1‴/C-5″ and C-7″ confirmed the prenyl group at C-6″ of the cinnamic acid moiety, and HMBC correlations from H-2′ to C-2″ connected this prenylated cinnamic acid moiety to the C-2′ hydroxy group of **6**. The absolute configuration of **3** was confirmed by co-injection of the alkaline hydrolysis products of **3** with an authentic sample of **6** on HPLC equipped with a chiral column. Thus, **3** was given the trivial name toddalin B.

Compound **4**, a colorless oil, gave the molecular formula $C_{32}H_{40}O_{12}$, as determined by ^{13}C NMR and HRESIMS data. The ^{1}H and ^{13}CNMR data (Table 2) of **4** were similar to those of **3**, with notable differences being the absence of an olefinic proton in **3** and the presence of two additional oxygenated carbons (δ_C 78.7 and 73.0) in **4**. HMBC correlations from two methyl singlets to the two oxygenated carbons along with the molecular formula suggested that the prenyl group was dihydroxylated in **4**. 2D NMR analysis established the molecular structure of **4**. Alkaline hydrolysis of **4** followed by acid cyclization yielded only a major product, which was identified as **6** by comparison with an authentic sample on a

Table 1. NMR Data for Toddalins A (1) and B (2) in Methanol-d_4 (δ in ppm)[a]

	1		2	
no.	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
2		163.0, C		163.1, C
3	6.12, d (9.6)	113.0, CH	6.13, d (9.6)	113.0, CH
4	7.89, d (9.6)	140.8, CH	7.89, d (9.6)	140.8, CH
5		157.7, C		157.6, C
6		117.9, C		117.9, C
7		163.7, C		163.6, C
8	6.64, s	96.5, CH	6.63, s	96.4, CH
9		156.5, C		156.4, C
10		108.3, C		108.3, C
1′a	2.92, dd (13.6, 2.3)	24.5, CH_2	2.92, d (13.6)	24.6, CH_2
1′b	3.20, dd (13.6, 11.2)		3.18, dd (13.6, 11.1)	
2′	5.28, dd (11.2, 2.3)	80.2, CH	5.26, d (11.1)	80.3, CH
3′		72.9, C		72.9, C
4′	1.31, s	26.0, CH_3	1.31, s	26.0, CH_3
5′	1.24, s	26.4, CH_3	1.24, s	26.4, CH_3
1″		76.9, C		76.9, C
2″a	1.77, d (13.6)	37.9, CH_2	1.76, d (14.4)	37.9, CH_2
2″b	1.96, dd (13.6, 2.1)		1.95, dd (14.4, 2.5)	
3″	4.05, m	71.5, CH	4.05, m	71.6, CH
4″	3.57, dd (9.4, 2.6)	73.8, CH	3.58, dd (9.3, 2.5)	73.7, CH
5″	5.21, ddd (10.0, 9.4, 4.7)	71.6, CH	5.21, ddd (9.8, 9.3, 4.5)	71.6, CH
6″a	2.0, m	39.5, CH_2	2.07, m	39.4, CH_2
6″b	1.46, dd (12.4, 11.3)		1.52, dd (12.2, 11.4)	
7″		174.2, C		174.2, C
1‴		127.7, C		127.7, C
2‴	7.20, s	111.7, CH	7.05, s	115.2, CH
3‴		149.4, C		146.8, C
4‴		150.8, C		150.0, C
5‴	6.82, d (8.2)	116.5, CH	6.80, d (8.2)	116.7, CH
6‴	7.07, d (8.2)	124.3, CH	6.96, d (8.2)	123.0, CH
7‴	7.57, d (16.0)	147.0, CH	7.51, d (16.0)	147.1, CH
8‴	6.26, d (16.0)	115.6, CH	6.18, d (16.0)	115.2, CH
9‴		168.5, C		168.6, C
5-OMe	3.86, s	63.8, CH_3	3.85, s	63.8, CH_3
7-OMe	3.87, s	56.8, CH_3	3.86, s	56.8, CH_3
3‴-OMe	3.93, s	56.5, CH_3		

[a] ^{1}H and ^{13}C NMR were recorded at 400 and 100 MHz, respectively.



1 3

Figure 1. Key HMBC (H→C) and ^{1}H–^{1}H COSY (—) correlations of **1** and **3**.

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Table 2. NMR Data for Compounds 3, 4, and 5 in $CDCl_3$ (δ in ppm)[a]

	3		4		5	
no.	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
2		161.6, C		161.4, C		161.3, C
3	6.10, d (9.6)	111.9, CH	6.10, d (9.6)	112.0, CH	6.13, d (9.6)	112.1, CH
4	7.73, d (9.6))	139.1, CH	7.75, d (9.6)	139.1, CH	7.75, d (9.6)	138.9, CH
5		156.1, C		156.2, C		156.1, C
6		117.0, C		117.0, C		116.9, C
7		162.1, C		162.1, C		162.1, C
8	6.49, s	95.3, CH	6.50, s	95.4, CH	6.52, s	95.4, CH
9		155.0, C		155.1, C		155.1, C
10		106.9, C		107.0, C		106.9, C
1′a	2.95, dd (13.6, 2.5)	24.0, CH_2	2.94, dd (13.5, 2.2)	24.1, CH_2	2.95, dd (13.7, 2.8)	24.2, CH_2
1′b	3.15, dd (13.6, 10.1)		3.15, dd (13.5, 10.1)		3.16, dd (13.7, 10.3)	
2′	5.30, dd (10.1, 2.5)	78.3, CH	5.30, dd (10.1, 2.2)	78.4, CH	5.31, dd (10.3, 2.8)	78.2, CH
3′		72.9, C		72.8, C		72.8, C
4′	1.38, s	25.1, CH_3	1.38, s	25.2, CH_3	1.38, s	25.2, CH_3
5′	1.34, s	26.5, CH_3	1.34, s	26.6, CH_3	1.34, s	26.7, CH_3
2″		168.8, C		168.5, C		168.4, C
3″	6.65, d (16.0)	117.0, CH	6.59, d (16.0)	117.8, CH	6.58, d (16.0)	117.7, CH
4″	7.78, d (16.0)	137.4, CH	7.67, d (16.0)	137.0, CH	7.70, d (16.0)	136.9, CH
5″		159.3, C		159.8, C		159.8, C
6″		115.6, C		112.7, C		109.5, C
7″		160.5, C		160.3, C		160.1, C
8″	6.21, s	95.7, CH	6.15, s	96.1, CH	6.20, s	96.0, CH
9″		156.9, C		157.5, C		157.9, C
10″		108.1, C		108.4, C		108.3, C
1‴a	3.20, d (6.4)	22.4, CH_2	2.57, dd (13.6, 3.3)	25.7, CH_2	3.70, s	35.7, CH_2
1‴b			2.80, dd (13.6, 10.1)			
2‴	5.09, t (6.4)	123.3, CH	3.50, m	78.7, CH		214.2, C
3‴		131.0, C		73.0, C	2.78, m	40.5, CH
4‴	1.64, s	25.6, CH_3	1.27, s	26.0, CH_3	1.16, d (6.9)	18.5, CH_3
5‴	1.72, s	17.7, CH_3	1.26, s	23.8, CH_3	1.16, d (6.9)	18.5, CH_3
5-OMe	3.84, s	63.2, CH_3	3.84, s	63.3, CH_3	3.84, s	63.3, CH_3
7-OMe	3.80, s	56.2, CH_3	3.82, s	56.2, CH_3	3.83, s	56.2, CH_3
5″-OMe	3.59, s	61.5, CH_3	3.60, s	61.5, CH_3	3.54, s	61.6, CH_3
7″-OMe	3.71, s	55.5, CH_3	3.67, s	55.6, CH_3	3.61, s	55.4, CH_3

[a] ^1H and ^{13}C NMR were recorded at 400 and 100 MHz, respectively.

chiral column. Thus, the absolute configuration of **4** was depicted as shown, and the compound was named toddalin C.

Compound **5** was obtained as a colorless oil. The molecular formula of **5** was found to be $C_{32}H_{38}O_{11}$ by ^{13}C NMR data and the HRESIMS ion at m/z 621.2303 $[M + Na]^+$ (calcd 621.2306). The ^1H and ^{13}C NMR data (Table 2) of **5** were similar to those of **3** except for the replacement of a prenyl group in **3** by a 3-methylbutan-2-one group in **5**. This was confirmed by HMBC correlations from the two methyl doublets [δ_H 1.16 (6H, d, J = 6.9 Hz, H-4‴ and H-5‴)] to a methine [δ_C 40.5] and a carbonyl carbon [δ_C 214.2]. The absolute configuration of **5** was defined by using the same method as for **3**. Compound **5** was given the trivial name toddalin D.

The HRESIMS data of compound **7** showed a molecular ion at m/z 493.1674 $[M + Na]^+$ (calcd 493.1680), which was 162 mass units more than that of **6**. The ^1H and ^{13}C NMR data of **7** were similar to those of **6** except for the presence of additional signals attributable to a β-glucopyranose residue [δ_H 4.56 (d, J = 7.7 Hz, H-1″) and δ_C 98.6 (C-1″)], indicating **7** was a glucosylated derivative of **6** or **15**. The glucopyranose moiety was located at C-3′ by HMBC correlation from H-1″ to C-3′, and this was supported by the deshielded C-3′ resonance (δ_C 81.9) in **7** compared to **6** (δ_C 72.8). Acid hydrolysis of **7** generated a coumarin/glucose mixture. The coumarin was confirmed as **6** by co-injection of the reaction mixture with an authentic sample on HPLC equipped with a chiral column. The D-configuration of the glucosyl unit was determined by HPLC analysis.[11,12] Thus, the structure of **7** was defined as (−)-toddalolactone 3′-*O*-β-D-glucopyranoside.

The known compounds toddacoumalone (**8**),[13] toddalosin (**9**),[9,14] 5-methoxyseselin (**10**),[15] braylin (**11**),[16] norbraylin (**12**),[17] toddaculin (**13**),[9] toddanone (**14**),[9] toddalolactone (**15**),[9] 5,7,8-trimethoxycoumarin (**16**),[18] coumurrayin (**17**),[9] gleinadiene (**18**),[19] *cis*-dehydrocoumurrayin (**19**),[20] toddalenone (**20**),[9] and toddacoumaquinone (**21**)[21] were identified by comparison of their observed and reported NMR data.

To enhance the structural diversity of the prenylated coumarin library for subsequent screening, compounds **10**, **11**, **13**, and **17** were modified to the corresponding hydrogenated analogues, 5-methoxydihydroseselin (**10a**),[22] 3′,4′-dihydrobraylin (**11a**),[23] 5,7-dimethoxy-6-(3-methylbutyl)-coumarin (**13a**),[9] and 5,7-dimethoxy-8-(3-methylbutyl)-coumarin (**17a**),[9] respectively. The library was screened for inhibitory activity against PED4D2 by using our reported methods.[24−27] Rolipram, a well-known PDE4 inhibitor, was

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used as the reference compound (IC_{50} 0.59 μM), comparable to the reported value of 1 μM.[1] The bioassay results showed that compounds **3**, **8**, **10**, **10a**, **11**, **11a**, **12**, **13**, **17**, and **21** had strong activity, with IC_{50} values less than 10 μM toward PDE4D2 (Table 3). The inhibitory curves of the two most active compounds (**8** and **11**, IC_{50} = 0.14 and 0.96 μM, respectively) are represented in Figure 2.

Table 3. IC_{50} Values of the Active Compounds against PDE4D2

compound	IC_{50} (μM)	compound	IC_{50} (μM)
2	16.65 ± 1.20	**12**	2.38 ± 0.14
3	7.81 ± 0.40	**13**	9.98 ± 0.63
8	0.14 ± 0.02	**13a**	11.49 ± 0.95
10	1.87 ± 0.12	**17**	6.82 ± 0.34
10a	2.20 ± 0.27	**17a**	10.22 ± 0.15
11	0.96 ± 0.10	**21**	5.51 ± 0.15
11a	1.53 ± 0.23	rolipram[a]	0.59 ± 0.05

[a]Positive control.

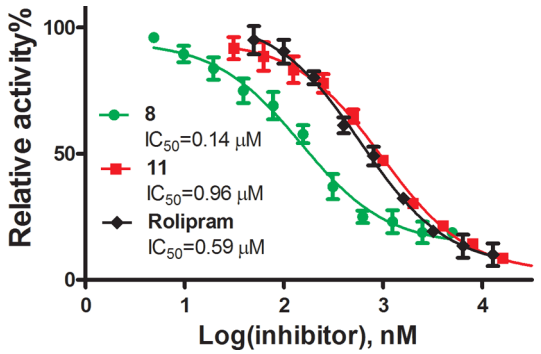

Figure 2. Inhibitory curves of compounds **8**, **11**, and rolipram (positive control) against PDE4D2.

It was found that compounds with an angular tricyclic system derived from the coumarin or quinolone core coupled with a pyran moiety exhibited the strongest inhibition, as exemplified by compounds **8**, **10**, **10a**, **11**, **11a**, and **12**; compounds with a nonoxygenated or nonconjugated prenyl moiety also showed good activity, e.g., **13**, **13a**, **17**, and **17a**. Thus, toddacoumalone (**8**), possessing both an angular tricyclic system and an unmodified prenylated moiety, showed the most potent activity. It is noteworthy that oxidation or conjugation of the prenyl moiety caused a significant decrease of the activity, such as in **1**, **2**, **4**, **5**, **14**, **18**, and **20**.

To further explore their inhibitory mechanism, the binding modes of **8**, **11**, and **12** with PDE4D were simulated by using the molecular docking approach CDOCKER.[28] The reliability of this method was validated by the root-mean-square deviation (RMSD) values for the top 10 redocked poses of roflumilast, which ranged from 0.8 to 1.5 Å relative to the crystal counterpart. It is considered that a successful docking holds the RMSD value of the optimum pose below a threshold of 1 Å in reference to the crystal pose.[22,23] Under identical conditions, compounds **8**, **11**, and **12** were docked into the PDE4D catalytic pocket, and the resulting poses for each ligand were ranged according to the "−CDOCKER_INTERACTION_ENERGY" scores. Several poses with high scores were further determined by the common scheme of inhibitors binding to PDE4, that is, the hydrophobic interactions and hydrogen bond interactions formed between the conserved residues (Phe372 and Gln369) and ligands.[29]

As shown in Figure 3, although these three active compounds comprised a similar angular tricyclic feature, the binding patterns of the simple coumarins **11** and **12** and compound **8** were different due to their reversed poses of the pyran ring in the tricyclic system. Compounds **11** and **12** could form two key hydrogen bonds with Gln369 via the oxygen atoms at C-6 and C-7 (2.9 Å/3.4 Å and 2.9 Å/3.2 Å, respectively) and generate

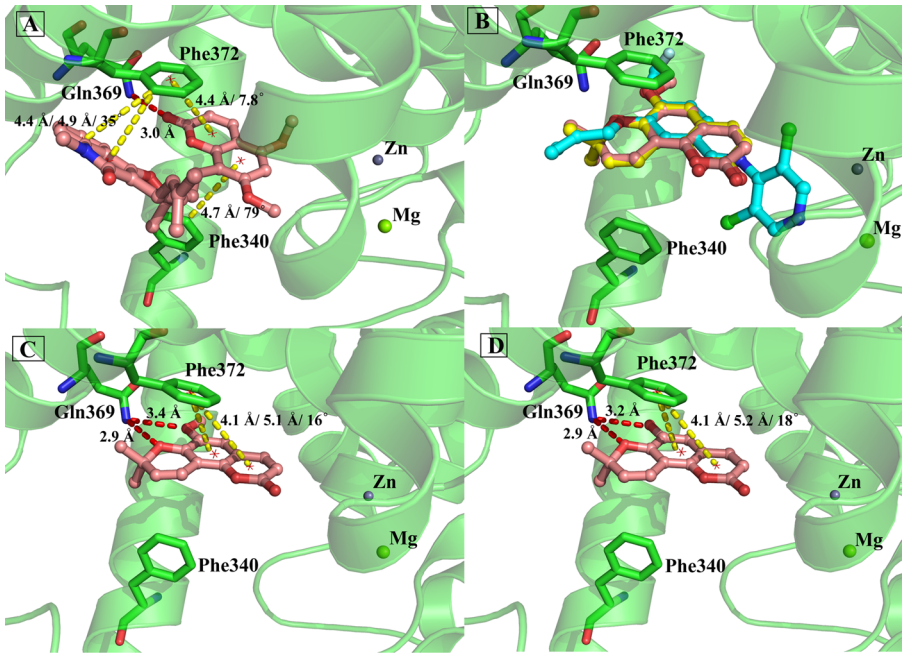

Figure 3. Binding modes of compounds **8**, **11**, and **12** with PDE4D derived from docking simulations (red dashed lines for hydrogen bond and yellow dashed lines for stacking interaction, respectively). (A) Binding mode of compound **8**. (B) The similar binding patterns of **11** (orange), **12** (yellow), and roflumilast (cyan). (C) Binding mode of compound **11**. (D) Binding mode of compound **12**.

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favorable stacking interactions with Phe372 via the 1-benzopyran-2-one ring system (4.1 Å/5.1 Å/16° and 2.9 Å/3.2 Å/18°, respectively, Figure 3), which shared a similar binding pattern to roflumilast (Figure 3B), as shown in the crystal structure of 1XOQ.[29] Interestingly, compound **8** forms only one hydrogen bond with Gln369 via the ester carbonyl group. However, the stacking interactions might be the predominant forces contributing to the binding of **8** with PDE4. For the coumarin moiety, it could form favorable interactions with hydrophobic residue Phe340 (4.7 Å and 79°) apart from interacting with the conserved Phe372 (4.4 Å and 7.8°). For the angular tricylic system, it could form two extra favorable stacking interactions with Phe372 (4.4 Å/4.9 Å and 35°), which might explain its relatively high inhibitory potencies despite the lack of one hydrogen bond. The absence of the pyran ring in **13**, **13a**, **17**, and **17a** decreased the stacking interactions between the ligands and Phe372, which led to a more moderate activity of this group of compounds, while their side-chain-modified analogues (**14**−**16** and **18**−**20**) lost the activity probably due to the steric effects caused by the oxygenated or conjugated prenyl tails (see Supporting Information).

Natural PDE4 inhibitors are rare, and the current study revealed a new group of PDE4 inhibitors from *T. asiatica*, which may explain the anti-inflammatory efficacy of this plant in Traditional Chinese Medicine. It is possible that the peculiar prenylated coumarin features confer on these compounds potent PDE4 inhibitory activity, which makes them promising lead structures for the development of PDE4 inhibitors. Studies toward their selectivity versus other PDE members are in progress.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Shimadzu UV-2450 spectrophotometer. IR spectra were determined on a Bruker Tensor 37 infrared spectrophotometer with KBr disks. NMR spectra were measured on a Bruker AM-400 spectrometer at 25 °C. ESIMS and HRESIMS were carried out on a Finnigan LC Q^{DECA} instrument. A Shimadzu LC-20 AT equipped with an SPD-M20A PDA detector was used for HPLC, and a YMC-pack ODS-A column (250 × 10 mm, S-5 μm, 12 nm) was used for semipreparative HPLC separation. A chiral column (Phenomenex Lux, cellulose-2, 250 × 10 mm, 5 μm) was used for chiral separation. Silica gel (300−400 mesh, Qingdao Haiyang Chemical Co. Ltd.), C_{18} reversed-phase silica gel (Rp-C_{18}) (12 nm, S-50 μm, YMC Co. Ltd.), Sephadex LH-20 gel (Amersham Biosciences), and MCI gel (CHP20P, 75−150 μm, Mitsubishi Chemical Industries Ltd.) were used for column chromatography (CC). All solvents used were of analytical grade (Guangzhou Chemical Reagents Company, Ltd.). Expression and purification of PDE4D were carried out by using a Hielscher UP200S ultrasonic cell disruption processor, a Sigma 6K15 centrifugal machine, an Eppendorf BioPhotomer spectrophotometer, and a Qiagen nickel-nitriloacetic acid (Ni-NTA) column. The radioactivity of the samples was measured on a PerkinElmer Tricarb 2910 liquid scintillation counter. The yeast extract and tryptone prepared for LB medium were purchased from Oxoid Ltd., and the substrate [^{3}H]-cAMP was from Waukesha GE Healthcare. Other reagents such as ampicillin and rolipram were purchased from Sigma.

Plant Material. Roots of *T. asiatica* were collected in October 2012 in Yunnan Province, P. R. China, and were authenticated by Prof. You-Kai Xu of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. A voucher specimen (accession number: FLZX201210) has been deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

Extraction and Isolation. The air-dried powder of the roots of *T. asiatica* (1 kg) was extracted with 95% EtOH (3 × 10 L) at room temperature (rt) to give 85 g of crude extract. The extract was suspended in H_2O (1 L) and successively partitioned with petroleum ether (PE, 3 × 1 L) and EtOAc (3 × 1 L), respectively. The EtOAc extract (63 g) was subjected to MCI gel CC eluted with a MeOH/H_2O gradient (3:7 → 10:0) to afford three fractions (I−III). Fraction I (10.5 g) was subjected to silica gel CC (PE/EtOAc, 2:1 → 0:1) to give three fractions (Ia−Ic). Fr. Ia (2.1 g) was separated by silica gel CC (PE/EtOAc, 2:1), followed by semipreparative HPLC equipped with a chiral column (CH_3OH/H_2O, 7:3, 3 mL/min), to give **6** (83 mg) and **15** (52 mg). Fr. Ic (5.3 g) was separated by Rp-C_{18} silica gel CC (MeOH/H_2O, 6:4 → 10:0) to yield **1** (65 mg), **2** (70 mg), and **7** (200 mg). Fraction II (36.5 g) was subjected to silica gel CC (PE/$CHCl_3$, 2:1 → 0:1) to give three fractions (IIa−IIc). Fr. IIa (4.1 g) was separated by Rp-C_{18} silica gel CC (MeOH/H_2O, 7:3 → 10:0) followed by Sephadex LH-20 CC using EtOH to give **10** (34 mg), **12** (21 mg), **13** (48 mg), **17** (37 mg), and **19** (54 mg). Fr. IIb (16.5 g) was subjected to silica gel CC (PE/$CHCl_3$, 1:1 → 0:1) to give three fractions (IIb_1−IIb_4). Fr. IIb_1 (2.9 g) was subjected to Rp-C_{18} CC (MeOH/H_2O, 6:4 → 10:0), followed by silica gel CC (PE/acetone, 12:1 → 0:1), to afford **11** (31 mg) and **14** (22 mg). Fr. IIb_2 (3.0 g) was applied to silica gel CC (PE/EtOAc, 8:1 → 0:1) and Sephadex LH-20 eluted with $CHCl_3$/MeOH, 1:1, to yield **16** (12 mg) and **18** (22 mg). Fr. IIb_3 (4.6 g) was subjected successively to silica gel CC (PE/EtOAc, 4:1 → 0:1), Rp-C_{18} CC (MeOH/H_2O, 6:4 → 10:0), and Sephadex LH-20 (EtOH) chromatography to yield **8** (32 mg), **9** (22 mg), **20** (17 mg), and **21** (15 mg). Fr. IIb_4 (3.2 g) was subjected to Rp-C_{18} CC (MeOH/H_2O, 6:4 → 10:0), silica gel CC (PE/acetone, 6:1 → 1:2), and Sephadex LH-20 (EtOH) to yield **3** (50 mg), **4** (10 mg), and **5** (6 mg).

*Toddalin A (**1**):* colorless oil; $[\alpha]_D^{20}$ −150 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.71), 222 (4.46), 327 (4.52) nm; IR (KBr) ν_{max} 3443, 1709, 1608, 1516, 1458 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 657.2 [M − H]$^-$, HRESIMS *m*/*z* 657.2191 [M − H]$^-$ (calcd for $C_{33}H_{37}O_{14}$, 657.2189).

*3‴-O-Demethyltoddalin A (**2**):* colorless oil; $[\alpha]_D^{20}$ −140 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.75), 222 (4.52), 329 (4.48) nm; IR (KBr) ν_{max} 3444, 1708, 1610, 1515, 1458 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 643.2 [M − H]$^-$; HRESIMS *m*/*z* 667.1991 [M + Na]$^+$ (calcd for $C_{32}H_{36}O_{14}Na$, 667.1997).

*Toddalin B (**3**):* colorless oil; $[\alpha]_D^{20}$ −116 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 250 (4.29), 325 (4.59) nm; IR (KBr) ν_{max} 3445, 1709, 1606, 1514, 1456 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; ESIMS *m*/*z* 581.3 [M − H]$^-$, HRESIMS *m*/*z* 605.2341 [M + Na]$^+$ (calcd for $C_{32}H_{38}O_{10}Na$, 605.2357).

*Toddalin C (**4**):* colorless oil; $[\alpha]_D^{20}$ −92 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 252 (4.16), 323 (4.45) nm; IR (KBr) ν_{max} 3446, 1709, 1615, 1454 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; ESIMS *m*/*z* 615.1 [M − H]$^-$; HRESIMS *m*/*z* 639.2432 [M + Na]$^+$ (calcd for $C_{32}H_{40}O_{12}Na$, 639.2412).

*Toddalin D (**5**):* colorless oil; $[\alpha]_D^{20}$ −107 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 251 (4.05), 324 (4.34) nm; IR (KBr) ν_{max} 3445, 1707, 1606, 1455 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; ESIMS *m*/*z* 597.2 [M − H]$^-$; HRESIMS *m*/*z* 621.2303 [M + Na]$^+$ (calcd for $C_{32}H_{38}O_{11}Na$, 621.2306).

*ent-Toddalolactone (**6**):* white powder; $[\alpha]_D^{20}$ −69 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 255 (3.94), 329 (4.11) nm; IR (KBr) ν_{max} 3446, 1610 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.79 (1H, d, *J* = 9.6 Hz, H-4), 6.58 (1H, s, H-8), 6.17 (1H, d, *J* = 9.6 Hz, H-3), 3.84 (3H, s, 7-OCH_3), 3.83 (3H, s, 5-OCH_3), 3.57 (1H, d, *J* = 10.2, 2.0 Hz, H-2′), 2.85 (1H, dd, *J* = 13.6, 2.0 Hz, H-1′b), 2.72 (1H, dd, *J* = 13.6, 10.2 Hz, H-1′a), 2.57 (OH, s), 2.32 (OH, s), 1.25 (3H, s, H-4′), 1.24 (3H, s, H-5′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 160.9 (C, C-2), 112.4 (CH, C-3), 138.7 (CH, C-4), 155.9 (C, C-5), 117.9 (C, C-6), 161.5 (C, C-7), 95.5 (CH, C-8), 154.8 (C, C-9), 107.1 (C, C-10), 26.0 (CH_2, C-1′), 77.7 (CH, C-2′), 72.8 (C, C-3′), 26.0 (CH_3, C-4′), 23.5 (CH_3, C-5′); ESIMS *m*/*z* 309.1 [M + H]$^+$; HRESIMS *m*/*z* 331.1152 [M + Na]$^+$ (calcd for $C_{16}H_{20}O_6Na$, 331.1158).

(−)-Toddalolactone 3′-O-β-D-glucopyranoside (7): colorless oil; $[\alpha]_D^{20}$ −44 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.53), 225 (4.16), 329 (4.07) nm; IR (KBr) ν_{max} 3444, 1609 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H 8.02 (1H, d, *J* = 9.6 Hz, H-4), 6.75 (1H, s, H-8), 6.23 (1H, d, *J* = 9.6 Hz, H-3), 4.56 (1H, d, *J* = 7.7 Hz, H-1″), 3.91 (3H, s, 7-OCH_3), 3.90 (3H, s, 5-OCH_3), 3.82 (1H, m, H-6″b), 3.82 (1H, dd, *J* = 10.2, 2.5 Hz, H-2′), 3.65 (1H, m, H-6″a), 3.40 (1H, m, H-3″), 3.30 (1H, m, H-5″), 3.29 (1H, m, H-4″), 3.21 (1H, m, H-2″), 2.91 (1H, m, H-1′b), 2.78 (1H, dd, *J* = 13.6, 2.5 Hz, H-1′a), 1.36 (3H, s, H-5′), 1.36 (3H, s, H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 163.4 (C, C-2), 112.6 (CH, C-3), 141.2 (CH, C-4), 157.7 (C, C-5), 120.2 (C, C-6), 163.7 (C, C-7), 96.3 (CH, C-8), 156.2 (C, C-9), 108.4 (C, C-10), 27.2 (CH_2, C-1′), 77.4 (CH, C-2′), 81.9 (C, C-3′), 21.8 (CH_3, C-4′), 23.9 (CH_3, C-5′), 98.6 (CH, C-1″), 75.2 (CH, C-2″), 78.1 (CH, C-3″), 71.7 (CH, C-4″), 77.7 (CH, C-5″), 62.7 (CH_2, C-6″); ESIMS *m*/*z* 493.1 $[M + Na]^+$; HRESIMS *m*/*z* 493.1674 $[M + Na]^+$ (calcd for $C_{22}H_{30}O_{11}Na$, 493.1680).

Acid Hydrolysis of 7 and Determination of the Absolute Configuration of Sugar and Aglycone. Compound **7** (2 mg) was refluxed with 2 mL of 2 M HCl (dioxane/H_2O, 1:1) for 4 h. After removing the dioxane under vacuum, the solution was diluted with H_2O and extracted with EtOAc (3 × 1 mL). The aqueous layer was evaporated under vacuum, diluted repeatedly with H_2O, evaporated under vacuum to obtain a neutral residue, and analyzed by TLC on silica gel (acetone/*n*-BuOH/H_2O, 6:3:1) with an authentic sugar sample (D-glucose, R_f = 0.49). The remaining residue was dissolved in pyridine (200 μL), to which 2 mg of L-cysteine methyl ester hydrochloride was added. The mixture was stirred for 1 h; 50 μL of *o*-tolyl isothiocyanate was added, and the mixture was stirred at 60 °C for another 1 h. The mixture was directly analyzed by standard C_{18} HPLC [a YMC-pack ODS-A column (250 × 10 mm, S-5 μm, 12 nm), CH_3CN/H_2O, 25:75, 3 mL/min]. The peak (t_R = 19.0 min) coincided with a derivative of D-glucose, as compared with authentic D-glucose with t_R at 19.1 min. In addition, the EtOAc layer was evaporated under vacuum to get the corresponding toddalolactone fragment, which was analyzed by HPLC equipped with a chiral column (CH_3OH/H_2O, 70:30, 3 mL/min). The *S* absolute configuration of the aglycone of **7** was confirmed by comparison of the retention time (t_R = 13.5 min) of the segments with that of (−)-toddalolactone in a similar way [(+)-toddalolactone t_R = 19.5 min and (−)-toddalolactone t_R = 13.5 min].

Hydrolysis of Compounds 1−5 and Determination of the Absolute Configuration of Constituent Units. Compounds **1**−**3** and **5** (each 2 mg) were refluxed with 4 mL of 1 M NaOH (MeOH/H_2O, 3:1) for 2 h. After cooling, each solution was neutralized with 1 mL of 1 M HCl, and the resin was removed by filtration. The filtrate was extracted with EtOAc to obtain the corresponding toddalolactone fragments, which were analyzed by HPLC equipped with a chiral column (CH_3OH/H_2O, 70:30, 3 mL/min). The *S* absolute configuration of the corresponding toddalolactone segments was confirmed by comparison of the retention time (t_R = 13.5 min) of the segments with that of (−)-toddalolactone [(+)-toddalolactone t_R = 19.5 min and (−)-toddalolactone t_R = 13.5 min]. Compound **4** (2 mg) was hydrolyzed under the above-mentioned basic conditions; then 4 mL of 2 M HCl was added. The mixture was refluxed until the phenylpropenoic acid moiety of **4** was converted to **6** as monitored by HPLC analysis.

Preparation of 11a, 12a, 13a, and 17a. To solutions of **10** (8.2 mg), **11** (5.8 mg), **13** (10 mg), and **17** (7.5 mg) in MeOH (4 mL) was added 10% Pd/C (0.8 mg). The mixtures were stirred under H_2 at rt for 1 h. The catalyst was filtered off, and the resulting products were subjected to flash chromatography eluting with MeOH/CH_2Cl_2 (20:1 → 10:1) to give **10a** (6 mg), **11a** (4.5 mg), **13a** (7 mg), and **17a** (5 mg), respectively. Analytical data: ^1H NMR spectra of compounds **10a**, **11a**, **13a**, and **17a** (see Supporting Information) were identical to reported data;[9,22,23] ESIMS *m*/*z* **10a** (261.1 $[M + H]^+$), **11a** (261.1 $[M + H]^+$), **13a** (277.1 $[M + H]^+$), and **17a** (277.1 $[M + H]^+$).

PDE4D Inhibitory Screening Assays. The protocols for expression, purification, and enzymatic assays of PDE4D2 were similar to those we described previously.[21−24] More details about the experimental procedures are provided in the Supporting Information.

Molecular Modeling. The crystal structure of the catalytic domain of human PDE4D2 with bound roflumilast (PDB code: 1XOQ[29]) was used here for the docking studies. The crystallographic water molecules were removed except those coordinated with the two metal ions Mg^{2+} and Zn^{2+}. Hydrogen atoms and charges were added to the systems using the CHARMm force field and the Momany-rone partial charge method, which were implemented in Accelrys Discovery Studio 2.5.5.[30] All ionizable residues in the systems were set to their protonation states at a neutral pH. The bound roflumilast was used as a reference compound to define the active site of PDE4 into which the active compounds were docked by using CDOCKER.[28] The radius of the input site sphere was set as 10 Å from the center of the binding site, and 10 random conformations were generated for each ligand. Other docking parameters were set to default values.

■ ASSOCIATED CONTENT

Supporting Information

This material (1D and 2D NMR spectra of **1**−**7**, 1D NMR spectra of **8**−**21**, expression and purification of PDE4D2, and enzymatic assays) is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(H.-B. Luo) E-mail: luohb77@mail.sysu.edu.cn.

*(S. Yin) Tel: +86-20-39943090 or +86-20-39943031. Fax: +86-20-39943090. E-mail: yinsh2@mail.sysu.edu.cn.

Author Contributions

†T.-T. Lin and Y.-Y. Huang contributed equally.

Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on March 5, 2014, with an error to Compound **17a** in the Results and Discussion section. The corrected version was reposted on April 2, 2014.